

A CONTRAVENTION OF ESTABLISHED PRINCIPLES OF INTERSPECIFIC
ALLOMETRIC METABOLIC SCALING IN DEVELOPING

SILKWORMS, *Bombyx mori*

Bonnie Blossman-Myer, B.S., M.S.

Dissertation Prepared for the Degree of
DOCTOR OF PHILOSOPHY

UNIVERSITY OF NORTH TEXAS

May 2007

APPROVED:

Warren Burggren, Major Professor and Dean
of the College of Arts and Sciences
Thomas Beiting, Minor Professor
Edward Dzialowski, Committee Member
Mark Burleson, Committee Member
Brian McMahon, Committee Member
Sam Atkinson, Chair of the Department of
Biological Sciences
Sandra L. Terrell, Dean of the Robert B.
Toulouse School of Graduate Studies

Blossman-Myer, Bonnie. A contravention of established principles of interspecific allometric metabolic scaling in developing silkworms, *Bombyx mori*. Doctor of Philosophy (Biology), May 2007, 115 pp., 3 tables, 28 figures, references, 122 titles.

Established interspecific metabolic allometric relationships do not adequately describe the complexity and variable physiological states of developing animals. Consequently, intraspecific allometric relationships of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) as a function of body mass; the respiratory quotient; the function of the silk cocoon; and body composition were investigated for each distinct developmental stage of the silkworm, *Bombyx mori*.

Whole animal $\dot{V}O_2$ in *Bombyx* ranged from 0.00064 ± 0.000047 ml $O_2 \cdot hr^{-1}$ at larval instar I to 0.77 ± 0.06 ml $O_2 \cdot hr^{-1}$ in pre-pupal, falling to 0.21 ± 0.01 ml $O_2 \cdot hr^{-1}$ in the pupae. Those instars having a significant relationship between $\dot{V}O_2$ as a function of body mass, the slope of the line relating $\dot{V}O_2$ to body mass varied between 0.99 and 1.02, while across all instars the slope was 0.82. Developmental allometry should be presented for individual developmental stages because the individual allometric exponents of the stages can be significantly different from the overall allometric exponent throughout development and in some cases, the overall allometric exponent can be a statistical artifact.

The first larval instar of *Bombyx mori* has the lowest cross sectional area of high metabolic tissue of the midgut (27%) and had one of the highest percentages of some metabolically inert tissues (i.e. lipid, 7.5%). Body composition of the first instar does not support the idea that smaller mass animals having the highest $\dot{V}O_2$ are composed of a greater percentage of metabolically active organs when compared to larger animals.

However, this developmental stage has the highest percentage of the mitochondrial marker cytochrome oxidase, which correlates well with the high $\dot{V}O_2$ rate of the smaller mass.

Therefore, established interspecific principles should not be assumed to function as valid models for intraspecific developmental relationships of metabolism as a function of body mass. Developmental allometry should include an analysis of individual stages of development as well as an analysis of development as a whole to gain a comprehensive understanding of the complexity of allometry of the developing animal such as the silkworm.

Copyright 2007

by

Bonnie Blossman-Myer

ACKNOWLEDGEMENTS

I would like to thank Dr. Warren Burggren for his superior mentoring and leadership. I would like to thank the lab manager Angie Trevino, and my lab mates Dao Ho, Greta Bolin, Matt Gore, Francis Pan, and Tara Blank. I would like to thank Dr. Ed Dzialowski, Dr. Tom Beitingger, Dr. Mark Burleson and Dr. Brian McMahon for serving on my committee. I wish to thank Dr. Pam Padilla for allowing me to use equipment in her laboratory. I would like to thank Dr. Norman Horner from Midwestern State University for assisting me with histological insect morphology. I wish to recognize my family – my husband Jason, daughter Whitney and son Zakk - for their love and tremendous support during the years.

This research was supported by NSF award number: 0128043.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vi
LIST OF FIGURES	vii
Chapter	
1. INTRODUCTION	1
Research Objectives	
Allometry and Scaling	
Metabolic Scaling	
Measuring Metabolic Rate	
Effect of Gender on Metabolic Rate	
Respiratory Quotient	
Body Compartmentalization	
Insects as Models	
The Silkworm, <i>Bombyx mori</i> as an Animal Model	
Gender Differences in <i>Bombyx mori</i>	
The Cocoon of <i>Bombyx mori</i>	
The Cytochrome System of <i>Bombyx mori</i>	
2. METABOLIC ALLOMETRY DURING LARVAL DEVELOPMENT AND METAMORPHOSIS OF THE SILKWORM, <i>Bombyx mori</i>	19
Introduction	
Materials and Methods	
Results	
Discussion	
Conclusion	
References	
3. METAMORPHOSIS, OXYGEN CONSUMPTION AND WATER LOSS IN THE SILKWORM, <i>Bombyx mori</i> : INVESTIGATION OF THE ROLE OF THE SILK COCOON.....	58

	Introduction	
	Materials and Methods	
	Results	
	Discussion	
	Conclusion	
	References	
4.	BODY COMPOSITION VARIATION DURING DEVELOPMENT OF <i>Bombyx mori</i>	78
	Introduction	
	Materials and Methods	
	Results	
	Discussion	
	Conclusion	
	References	
5.	CONCLUSION.....	101
	REFERENCES.....	106

LIST OF TABLES

	Page
2.1 Respirometry variables for oxygen consumption measurements	25
3.1 Mean $\dot{V}O_2 \pm$ s.e. of control (intact cocoon), sham (removed from cocoon and reinserted into cocoon) and naked pupae (removed from cocoon) of metamorphosing <i>Bombyx mori</i> pupae at $25 \pm 0.5^\circ\text{C}$	69
3.2 Body mass loss of intact and naked pupae (removed from cocoon) and empty cocoons of metamorphosing <i>Bombyx mori</i> pupae over time at a low relative humidity (30-35%) and at $24 \pm 1.0^\circ\text{C}$	71

LIST OF FIGURES

	Page
2.1 Life cycle of <i>Bombyx mori</i>	22
2.2 Oxygen consumption schematic diagram.....	24
2.3 Body mass of developing <i>Bombyx mori</i> as a function of developmental stage	29
2.4 Semi log plots of (a) whole animal $\dot{V}O_2$ of developing <i>Bombyx mori</i> as a function of body mass and (b) mean whole animal $\dot{V}O_2$ as a function of body mass	31
2.5 Double log plots of (a) whole animal $\dot{V}O_2$ and, (b) mean whole animal $\dot{V}O_2$ and (c) whole animal $\dot{V}O_2$ as a function of body mass in developing <i>Bombyx mori</i> . 33	33
2.6 Semi log plot of mass specific $\dot{V}O_2$ as a function of body mass of developing <i>Bombyx mori</i> and (b) mean mass specific oxygen consumption as a function of developmental stage of developing <i>Bombyx mori</i>	35
2.7 Double log plot of mass-specific $\dot{V}O_2$ and (b) mean mass specific $\dot{V}O_2$ and (c) mass specific $\dot{V}O_2$ as a function of body mass across development of <i>Bombyx mori</i>	37
2.8 Double log plot of mean (a) whole animal and (b) mass specific $\dot{V}O_2$ as a function of body mass by gender in developing <i>Bombyx mori</i>	39
2.9 Double log plot of (a) mean whole animal $\dot{V}O_2$ and (b) mean mass-specific $\dot{V}O_2$ as a function of total surface area of developing <i>Bombyx mori</i>	41
2.10 Whole animal $\dot{V}CO_2$ and (b) mass-specific $\dot{V}CO_2$ as a function of body mass of developing <i>Bombyx mori</i>	43
2.11 Double log plot of (a) whole animal and (b) mass-specific carbon dioxide production as a function of body mass in developing <i>Bombyx mori</i>	45
2.12 Mass specific $\dot{V}O_2$ and $\dot{V}CO_2$ and (b) respiratory quotient values as a function of developmental stage of <i>Bombyx mori</i>	46
2.13 Heart rate as a function of body mass in developing <i>Bombyx mori</i>	47
3.1 Body mass throughout the life cycle of <i>Bombyx mori</i>	65
3.2 Rate of body mass change by developmental stage of <i>Bombyx mori</i>	66
3.3 $\dot{V}O_2$ as a function of body mass of prepupal, pupal and adult <i>Bombyx mori</i>	68

3.4	Whole animal $\dot{V}O_2$ and (b) mass-specific $\dot{V}O_2$ of control (pupa plus cocoon), sham (opened cocoons), and naked pupa of metamorphosing <i>Bombyx mori</i> ..	69
3.5	Whole animal and (b) mass-specific $\dot{V}O_2$ as a function of body mass in developing <i>Bombyx mori</i> in intact pupae (with cocoon mass) and with naked pupae (minus cocoon mass).....	70
3.6	Percentage of body mass change over time of three experimental groups (control, naked and empty cocoons) of metamorphosing <i>Bombyx mori</i> held in constant dry relative humidity of 30-35% and 23-5°C.....	72
3.7	Rate of body mass of control pupae, naked pupae, and empty cocoons of <i>Bombyx mori</i> over time held in constant dry relative humidity of 30-35% and 23-25°C	73
4.1	Histological light microscopy cross section of a larval instar I <i>Bombyx mori</i> with morphology labeled	83
4.2	Total body surface area as a function of body mass of developing <i>Bombyx mori</i>	88
4.3	Surface area of cross sections of (a) total body and midgut of <i>Bombyx mori</i> by body mass	89
4.4	Midgut cross section surface area (sa) as a percentage of total cross section sa as a function of body mass in <i>Bombyx mori</i> by developmental stage.....	90
4.5	Mean ratio of the (a/d) cuticle, (b/e) silk gland and (c/f) midgut to total body mass for each developmental stage of <i>Bombyx mori</i>	92
4.6	Body water content as a percentage of total body mass in <i>Bombyx mori</i> as a function of developmental stage	93
4.7	Lipid content as a percentage of (a) wet body mass and (b) dry body mass in <i>Bombyx mori</i> by developmental stage.....	94
4.8	Cytochrome oxidase activity per gram of body mass by developmental stage of <i>Bombyx mori</i> silkworms	95

CHAPTER 1

INTRODUCTION

Research Objectives

In the first study (Chapter 2), I describe the oxygen consumption rate ($\dot{V}O_2$) and the carbon dioxide production rate ($\dot{V}CO_2$) during each stage of the lifecycle of *Bombyx mori* using closed respirometric techniques from larval instar I through adults to determine if oxygen consumption rates as a function of body mass followed the interspecific allometric curve established for adults. In this study (Chapter 2), I hypothesized that the intraspecific metabolic rate of a silkworm over the lifecycle would not follow the same allometric pattern as interspecific adults, would not differ by gender, and that the RQ value of the silkworms would not differ from 1.0. I also analyzed the data in this Chapter and in Chapter 4 to determine the most appropriate method for data analyses and presentation for intraspecific developmental metabolic allometric relationships.

In the second study (Chapter 3), I describe $\dot{V}O_2$ during metamorphosis of the silkworm, *Bombyx mori* to determine if the cocoon formed during metamorphosis is a limiting factor of either the rate of $\dot{V}O_2$ and / or of body mass via water loss. I hypothesized that the cocoon reduces the diffusion rate of oxygen and limits the rate of water loss during metamorphosis.

In the third study (Chapter 4), I describe histological and gross morphology, percentage of body water and lipids, and cytochrome oxidase activity levels of developing *Bombyx mori* silkworms to determine the morphological and biochemical

body compositions by developmental stage. I hypothesized that larval instar I – with the lowest body mass and the highest mass specific $\dot{V}O_2$ – would have the highest amount of metabolically active tissues per gram of body mass as indicated by histological, gross morphological, and biochemical measurements. To perform the research described an understanding of allometry and scaling, metabolism, insect development and metamorphosis and body compartmentalization was essential.

Allometry and Scaling

The surface area of a cell is the critical interface between the organism and its environment. Exchange of materials across this interface often occurs through the process of diffusion, in which dissolved molecules or other particles move from areas of higher concentration to areas of lower concentration (although some exchange is mediated by active transport). Diffusion is a passive process, and as a result imposes constraints upon the size of a single-celled organism or cell. Materials must be able to reach all parts of a cell quickly, and when volume is too large relative to surface area, diffusion cannot occur at sufficiently high rates.

An appreciation of dimensional changes is key to understanding allometric relationships. If two objects are isometric, their areas are proportional to ℓ^2 and Volumes proportional to ℓ^3 . The slope of the line is equal to 2/3 or 0.67, a relationship known as the Surface Law. The relationship of surface area to volume determines how size influences the structure of organisms.

While isometric growth describes size-related changes in many inanimate objects, isometric growth does not typically occur in animals. If an elephant, for example, grew isometrically, it would be unable to support itself on land as its bones

would not have grown thick enough to support its body mass. For an elephant's structure to support its weight, its bones have to thicken and grow at a faster rate than its other body structures. Disproportional relationships between body mass and other variables (e.g. metabolic rate) usually form a curved line. Allometric relationships can be presented as a linear graph by either data transformation or by using a double logarithmic plot. Allometric relationships can be studied during development of a single organism, during intraspecific development, and for interspecific comparisons.

Allometry, which can be used for comparative research, is the study of the development or growth of one part of an organism in relation to another, with parts growing at various rates. Allometry is *body - size relationships* and allometric relationships set the parameters for scaling relationships (Peters, 1983). Scaling is the change of a specified trait in relation to body mass. This relationship is best expressed by Huxley's bivariate allometric equation: $Y=aM^b$, where Y is the trait being studied, M is the body mass of the animal and a is a species-specific proportionality coefficient. The proportionality coefficient varies with both species and the type of trait. The allometric exponent describes the relationship of the two traits being studied. Not only are allometric relationships within an organism used for comparison, allometric scaling allows for interspecific evaluations.

Metabolic Scaling

Metabolic rate controls all physiological aspects of an organism, therefore the scaling of metabolic rate as a function of body mass is one of the most studied allometric relationships (Darveau et al., 2002; West et al., 2002; White and Seymour, 2002; Hochachka et al., 2003; Agutter and Wheatley, 2004; Hunt Von Herbing, 2006;

Long et al., 2006, and others). Metabolic scaling can be affected by multiple variables such as the level of activity or stress, environmental conditions and the stage of development (Schmidt-Nielson, 1984; Burggren, 2005; Hunt Von Herbing, 2006). In 1932, Kleiber reported that an allometric exponent of 0.75 applies for metabolic scaling relationships with body size in mammals, and this is known as “Kleiber’s Law”. Kleiber (1932) published data contributing to the “mouse to elephant curve” later created by Brody (1945). One problem with the “mouse to elephant curve” was that there were assumptions made that all animals measured had analogous anatomy and physiological states, and that standard laboratory conditions applied to all measurements of metabolic rate (Hunt Von Herbing, 2006). A mouse clearly does not possess identical anatomy or physiology to an elephant. Later, the allometric exponent of 0.75 relating metabolic rate and size was found to apply to poikilothermic organisms (Wieser, 1984). Heusner (1982) challenged the 0.75 scaling exponent of Kleiber (1932) by declaring it a statistical artifact. Heusner (1982) argued that the slope of 0.75 was made up from individual slopes having values closer to 0.67 and Feldman and McMahon (1983) disagreed with Heusner and defended Kleiber’s law. This began the controversy over the 0.75 (Kleiber’s law) and 0.67 (Surface law) scaling exponents of metabolic rate and body mass. Wieser (1984) stated that the source of confusion may be due to the lack of distinction between intraspecific and interspecific allometry or between metabolic allometry using whole animal metabolic rate versus mass specific metabolic rate. White and Seymour (2003) reported that mammalian metabolic rate allometrically scales by the Surface Law and that the quarter power scaling law is flawed because animals measured are not in similar physiological states such as activity level, specific dynamic

action, circadian rhythms, environmental temperature, and developmental and reproductive stages; and it does not take into account any variations in body temperature.

There has not been an overall accepted rationalization found for this universal scaling exponent of 0.75, therefore it remains a questionable biological phenomenon. However, there have been possible explanations reported such as the mammalian model by West et al. (2002) based on vascular networks and energy pathways that attempted to explain the universal 0.75 scaling exponent for all organisms as well as for other levels of organization such as cells and even mitochondria. This model is reported to hold true for insects as the fractal-like network is considered to be the tracheal system (West et al., 2002). Darveau et al. (2002) reported an “allometric cascade” model consisting of many contributors to a whole organism metabolic rate with each contributor having an individual and variable allometric exponent (Darveau et al., 2002; Hochachka et al., 2003). Darveau et al. (2002) also made the determination that basal metabolic rate and maximum metabolic rate have differing scaling coefficients. Basal metabolic rate scales closer to Kleiber’s law and depends on the needs for energy, i.e. protein synthesis, ion pumping, ATP synthesis; whereas maximal metabolic rates scale higher (~ 0.7 to 1.0) and depend on the providers of energy, i.e. ventilation, diffusion of gases, cardiac output, and gas exchange at the capillaries (Burness, 2002).

Developmental Allometry

Developing animals may not follow the general allometric guidelines and models that are established for interspecific adult comparisons such as the direct relationship of an increased mass with an increased metabolic rate. Instead, animals may reveal

complex allometric patterns during development (Smith, 1984; Burggren, 2005; Frankimo et al., 2005; Hunt Von Herbing, 2006). Complex patterns, such as fluctuations in the relationship of metabolic rate and body mass over development, could result from changes at the cellular level. These cellular changes could occur when the developing animal switches from experiencing the metabolic costs of mitosis to the lower metabolic costs of maintaining those tissues (Kozlowski, 2003). Interspecific allometric relationships between adults have for the most part, been established. However, intraspecific developmental allometry is infrequently investigated and should reveal much about both allometry and development. Growth rates vary intraspecifically during the life cycle of an animal with the most rapid growth occurring during the first periods of the animal's life. Several questions arise. Do developing organisms follow the same allometric relationships as the adults of the same species? Do allometric relationships fluctuate during the developmental stages? Once the general allometric relationships of the developing organism of each species are established, the stage is set for the more advanced questions to be answered as to why and how these relationships exist.

Ontogenic allometric homeothermic models have previously been established that incorporate whole animal metabolic rate, average individual cell metabolic rate, and metabolic rate needed for mitosis (West, 2001). There are examples of how organisms can have variable metabolic rates as a function of body mass throughout development. One example is with mass-specific human phases of metabolism in which the fetus resembles a maternal organ, the neonate mimics maternal metabolic rate, followed by intensive growth phase that scales isometrically with a subsequent maintenance phase scaling at 0.66 and a final senescent declination

of metabolic rate (Wieser, 1984). Clearly, in this example, there is not one universal allometric equation to describe the relationship of metabolic rate to body mass, but rather the relationships are better described by developmental stages throughout the life cycle.

Measuring Metabolic Rate

Metabolism is the transformation of the assimilated energy consumed by an organism and the storage and expenditure of that transformed energy. It is a fundamental biological process and its rate is a solid indicator of the physics, biochemistry and the physiology of an organism. Measuring the rate of oxygen consumption ($\dot{V}O_2$) is an accepted indirect method of estimating aerobic metabolic rate in animals and has been used in many allometric studies (Fink, 1925; Frew, 1927; Kleiber, 1932; Brody, 1945; Watanabe and Williams, 1950; McMahon, 1973; Wood et al., 1978; Tenney, 1985; German, 1989; West, et. al, 1997; West, 2002; Kozlowski, 2003; Marais and Chown, 2003; Brown, 2004; Economo, 2005, and others). Oxygen consumption can be expressed as whole animal ($\text{ml O}_2 \cdot \text{hr}^{-1}$) or mass-specific ($\text{ml O}_2 \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$). If the organism follows the quarter power scaling law, the mass-specific metabolic rate decreases with increasing body mass, and expresses a negative slope value of $-0.24 - 0.25$ on a double logarithmic graph (Couture, 1995; West, 1997). When the mass of an adult mammal increases twofold, the mass specific metabolic rate declines by 15% (Couture, 1995). Therefore, per unit mass, a smaller animal has a higher metabolic rate.

Effect of Gender on $\dot{V}O_2$

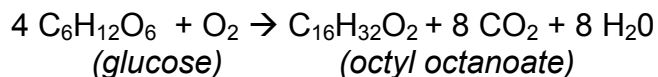
When measuring oxygen consumption, intraspecific gender differences should be considered. In some species, gender can influence oxygen consumption rates. From cockroaches to humans there are significant gender differences in resting metabolic rates (Hostetler et al., 1994; Poehlman, et. al, 1997; Schutz, 1997; Rogowitz, 2000; Nespolo et al, 2003). Most of these studies were performed on adults having different body compositions – i.e. on the average; a human female has a higher percentage of fat and lower percentage of protein than a similar sized male.

Respiratory Quotient

The energy source fueling metabolism should also be considered when comparing metabolic rates. The respiratory quotient (RQ) gives information about the source of energy that an animal is using for metabolism. The RQ is defined as the amount of carbon dioxide produced relative to the amount of oxygen consumed. According to Avogadro's hypotheses, if oxygen and carbon dioxide act as ideal gases, the RQ can be expressed as a ratio of volumes (Kleiber, 1975). An aerobic organism that is burning carbohydrate energy sources has equal rates of oxygen consumption as carbon dioxide production and has an RQ of 1.0. The same organism during fat catabolism produces an average ratio of 6.3 moles of carbon dioxide to the consumption of 8.9 moles of oxygen to give an RQ of 0.71 (Kleiber, 1975). It is not as straightforward to determine protein consumption, but an RQ between 0.74 to 0.81 is considered to be an indicator of protein catabolism, but may also be an indicator of a mixture of fat and carbohydrate catabolism as well (Kleiber, 1975; Schmidt-Neilson,

2001). Protein catabolism varies because protein is not entirely oxidized during catabolism and the nitrogen from amino acids is excreted from organisms.

Normal RQ values range from 0.70 to 1.0, but there many instances where RQs have been recorded above and below this range. One reason an RQ can occur below 0.70 is due to the synthesis of carbohydrates via gluconeogenesis (Kleiber, 1975). Other researchers have also found RQ's below 0.70 in insects during metamorphosis as well in other animals and some have compared this state in insects to hibernating mammals (Walsburg and Hoffman, 2005). RQ values above 1.0 have also been described. Lusk, 1917, delineated a biochemical formula with an RQ value of 8.0 that would occur from the synthesis of a fat (octyl octanoate) from glucose (Kleiber, 1975):



Klieber (1975) found an RQ of 1.33 in geese that consumed large amounts of grain. He accounted for the increase in RQ by the synthesis of fat from carbohydrates. Another example of an RQ found to be greater than one is in a study involving mammary tissue slices. These tissues slices had an RQ above 1 due to fat synthesis (Popjak, 1957).

The RQ is not a definitive tool used to pinpoint metabolic energy sources. For example, in humans, up to about four liters of carbon dioxide can wash out from the carbonate buffer system twice every hour and this liberation of carbon dioxide is not a metabolic by-product but causes the RQ to inflate erroneously (Klieber, 1975). Fasting organisms before measurements are made to select the material source for metabolism is also an unreliable method. Animals assumed to have been fasted by researchers were found to catabolize carbohydrates that were stored in the gut (Walsberg and Hoffman, 2005). Therefore, the RQ is a tool for tracking changes in metabolism or as a

starting point for further investigation of the energy sources of metabolism. However, RQ as a quantification of metabolism energy sources should be used with caution.

Body Compartmentalization

Metabolic allometric relationships set the stage for more advanced questions to be investigated, such as - what are the causes of metabolic rate fluctuations?

An animal body can be subdivided into five major classes: atomic, molecular, cellular, tissue-system and the whole body - with each characterized by allometric relationships between metabolic rate and body mass (Wang et al., 1992). Organisms can be compartmentalized into lean body mass (metabolically active) and fat body mass (metabolically inert) with lean body mass having a stronger relationship to metabolic rate than the whole animal mass (Wang et al., 1992; Hunt Von Herbing, 2006). The proportion of lean body mass determines the metabolic rate and larger animals have a lower mass-specific $\dot{V}O_2$ due to an increasing percentage of metabolically inert tissue. The individual $\dot{V}O_2$ of organs from higher body mass animals is lower than that of smaller body mass animals (Wang et al., 1992; Hunt Von Herbing, 2006). This may be due to an increase in metabolically inert tissue, i.e. adipose, as opposed to gains in metabolically active tissue mass, i.e. muscle. The mass of metabolically active tissues per body mass is variable as is the $\dot{V}O_2$ as a function of body mass (Schmidt-Nielsen, 1984; Wang et al., 1992; Hulbert and Else, 2005). All animals can be viewed as having multiple body compartments with varying degrees of energy consumption. Organs with the highest metabolic rates may not increase in the same proportion with increasing size as the organs with lower metabolic rates, such as adipose tissue (Hulbert and Else, 2005; Hunt Von Herbing, 2006). Larger vertebrates were shown to have equivalent

percentages of skeletal muscle of the total body mass as compared to smaller vertebrates (Hunt Von Herbing, 2006). However, larger vertebrates were shown to have a higher percentage of metabolically passive tissue, i.e. lipids and bone tissue, of the total body mass as compared to smaller animals and a smaller percentage of metabolically active tissue, i.e. nervous tissue and liver (Hunt Von Herbing, 2006). One of the highest metabolically active tissues in the body is muscle – especially the flight muscles of insects (Weis-Fogh, 1964; Downer and Matthews, 1976; Suarez et al., 2005). The midgut is another highly active organ with a mitochondrial and rough endoplasmic reticulum rich columnar epithelial layer and outer circular and longitudinal smooth muscle layers (Shultz and Jungreis, 1977; Thomas and May, 1984; Baldwin and Hakim, 1991; Gibellato and Chamberlin, 1994). The goblet cells are dispersed throughout the columnar epithelium and also are rich in mitochondria and play an active role in ion transport (Shultz and Jungreis, 1977; Thomas and May, 1984; Baldwin and Hakim, 1991; Gibellato and Chamberlin, 1994). The silk glands of *Bombyx mori* should be considered a high metabolically active organ as its primary function is the synthesis and secretion of silk proteins which requires energy (Morimoto et al., 1968; Tashiro et al., 1968; Sasaki et al., 1976, Sasaki et al., 1981). Heart and nervous tissue were shown to have high metabolic rates (Hunt Von Herbing, 2006). By comparison, the cuticle and total body lipid content should be considered low metabolically contributing tissues (Schmidt-Nielsen, 1984; Hunt Von Herbing, 2006).

Water does not directly contribute to aerobic respiratory rates. As with water, the total body lipid content should be considered to be in the metabolically inert group of body compartments. In insects, lipids primarily serve as cellular membrane structure

and as precursor materials of the steroidal hormone, ecdysone. The most common lipids are phospholipids, sphingolipids, steroids, and glycerides - which make up approximately 90% of lipids within an insect body (Fast, 1964; Patel et al., 2004; Canavoso et al., 2004). Most lipids in the insect body are stored in a dispersed organ termed the fat body. The fat body is analogous to both the liver and adipose tissue in vertebrates as it has similar functions such as energy storage, production of hormones and proteins, and the storage of glycogen and in insects, it would be considered a highly metabolic organ (Canavoso et al., 2004; Patel et al., 2004). A major change in the fat body during the prepupal stage of metamorphosis is the switch from active lipid storage to the utilization of lipid reserves which is most likely caused by a peak in ecdysone (Prasad et al., 1986).

Multiple reasons may explain why smaller animals have a higher metabolic rate per unit mass. One reason may be the larger number of mitochondria in the muscle cells of smaller animals as compared to the larger animals (Schmidt-Nielsen, 1984). Else and Hubert (1981) found that the mice (*Mus musculus*) with the highest metabolic rate had a greater volume of tissue dedicated to mitochondria than lizard (*Amphibolurus nuchalis*) tissues.

There are two ways that the size of an organism can increase – by cell size or number. During early development, hyperplastic growth is predominant over hypertrophic growth. Hyperplasia is mitotic growth and hypertrophy is the growth of individual cell mass. There might be a window or multiple windows during development in which these two cellular processes switch roles and hypertrophy becomes the major route of size increase. In insects, there is a hyperplastic surge at the end of the larval

instars due to the growth of a new cuticle and this surge is associated with a rise in $\dot{V}O_2$ (Rockstein, 1973). Both of these growth types need to be considered when considering metabolic scaling during development. Kozlowski (2003) considered the role of C values, a quantification of the genome size in a cell, as a factor in scaling. He stated that the amount of DNA in a larger cell is primarily non-coding sequences. Kozlowski (2003) found that smaller cells tend to have higher metabolic rates and established an inverse relationship between the C value and metabolic rates between taxonomic groups. As a result, as cells increase in size, there is only a Volume increase and not a metabolic rate increase and this may be yet another cause for smaller animals having a per unit mass higher metabolic rate.

Insects as Models

There is vast metabolic allometric literature in vertebrates (Kleiber, 1932; Brody, 1945; McMahon, 1973; Batterham et al., 1999; Brown et al., 2004; Agutter and Wheatley, 2004; Economo, 2005, and others); however, there are relatively few developmental metabolic allometric studies of insects. This is surprising as insects are the largest and most diverse class of animals. Allometric investigation of insects could also reveal relationships over the course of development that are analogous to vertebrate systems. Insects have been primarily used throughout the years for genetic research – especially the fruit fly, *Drosophila melanogaster*. However, other types of insect research, such as biochemical research, can also reveal applicable data for vertebrate systems as there are many analogous tissues and biochemical pathways (Law and Wells, 1989; Szolajaska et al., 2004). The brief life cycle of insects can provide large populations necessary for biochemical research (Law and Wells, 1989).

Historically, to a lesser degree than genetic research, insects have been utilized in biochemical research. Ecdysone was one of the first compounds to be investigated in steroidal pathways (Law and Wells, 1989). Insect research in proteolytic cascades, lipid delivery systems, and ligand systems has made important contributions to biochemistry literature (Law and Wells, 1989).

The Silkworm, *Bombyx mori* as an Animal Model

Attributes of an animal model that make it useful for intraspecific studies of metabolic allometry include specific and externally evident morphological markers for development, as well as large body mass changes over the life cycle. The silkworm, *Bombyx mori*, has these attributes. During development, the *Bombyx mori* body mass spans from larval instar I to adult moth over approximately four orders of magnitude (body mass increase from ~ 0.0004 g to 2.2 g). The 55-60 day life cycle of *Bombyx mori* begins with the egg. The larva hatches, i.e. eclosion, in about ten days. This is followed by a 27-day larval stage marked by five distinct instars separated by ecdysis, or molting of the outer cuticle.

During molting, the larva produces a silk pad that adheres its ventral cuticle to the surface. The larva remains still for up to one day while ecdysone (a molting hormone) levels rise and a new cuticle is formed underneath the layers of the old one (Reynolds, 1980; Ganga, 2003; Emlen and Allen, 2004). Larval to larval molts only occur when juvenile hormone (JH) is present (Rockstein, 1973; Ganga, 2003, Emlen and Allen, 2004). When ecdysone reaches a homeostatic set point, the larva increases the pressure inside of the body and bursts out of the old cuticle and commences feeding. During the fifth larval stage, approximately 25 days after hatching, the silkworm

undergoes a period of intense feeding. In response to a surge of ecdysone and the absence of juvenile hormone, the larva wanders and this marks the beginning of the prepupal stage and hence, metamorphosis. For approximately 5 days, the prepupa spins a silk cocoon around its contracted body and tanning of the outer cuticle occurs. Histolysis and histogenesis occur simultaneously during metamorphosis. The silk glands are the first to undergo histolysis, possibly from programmed cell death (apoptosis and phagocytosis) (Ohtaki, 1986). The proliferation of imaginal tissues of the wings, legs, antennae, thorax and abdomen are followed by the histolysis of the midgut and the mandible (Ohtaki, 1986). This is the developmental stage where embryogenesis-formed imaginal tissues undergo development to form the adult morphology. Once metamorphosis is complete, the adult secretes a digestive enzyme that dissolves a hole in the silk cocoon large enough for emergence, i.e. eclosion. The adults have minimal mouthparts and therefore cannot consume food (Rockstein, 1973; Ganga, 2003). They must survive the next five days or so to reproduce on energy stored during the final larval stage.

Gender Differences in *Bombyx mori*

Gender differences must be established in order to determine significant differences in metabolic rate during development. There are morphological gender differences in silkworms that appear as early as the fourth instar. Female silkworms at or beyond larval instar IV typically have a body mass larger than males. This should not be used as an indicator of gender, however, because this is not always the case. There are sexual differences found on the pupal cases. The female pupa has a larger, wider abdomen and the male is slender and more tapered. The female has a ventrally located

vertical line between the 8th and 9th segment whereas the male has a round dot on the 9th segment. Adult *Bombyx* are different morphologically as well as behaviorally. The males have more pronounced antennae, are usually smaller, and are more active. The males also have a pair of hooks as part of their external genitalia that are used to hold the female during sexual intercourse. The females have a bulb-like gland that protrudes from the abdomen to release male-attracting pheromones. The observed elevated activity of males, i.e. vigorous shaking, rapid movement, may be due to the stimulus of the receptors on the antennae caused by the female pheromones - bombykol and bombykal. The detection of female pheromones is the function of more than 17,000 sensilla on the male antennae (Syed et al., 2006). Therefore, the sex of the silkworm can be determined during metamorphosis by viewing nearly microscopic morphological characteristics and during the adult stage by noting larger and more obvious morphological differences as well as behavior differences.

The Cocoon of *Bombyx mori*

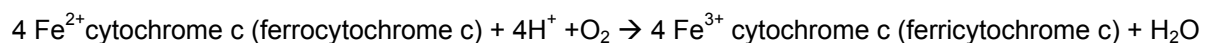
When measuring oxygen consumption of metamorphosing silkworms, it is beneficial to know the function of the cocoon and its effects on metabolic rate. The silkworm secretes silk from labial glands that are analogous to salivary glands in other larval insects. Inside of the silk gland lumen, the silk remains gelatinous and during secretion, the silk polymer dehydrates and strengthens (Sutherland et al., 2006). The high tensile strength characteristics of silk make it desirable for use in biomedical tissue engineering (Hakimi, et al., 2006). Silk is made up of two major components - microfilaments of an insoluble protein (fibroin), covered with a soluble adhesive protein (sericin) that provides structural support to the cocoon (Hakimi et al., 2006). Fibroin

mainly consists of a 390 kDa heavy polymer (H-fibroin) and two types of 25 kDa light polymers (L-fibroin) linked by disulfide bonds with alternating hydrophilic and hydrophobic sheets that increase the resilient characteristics of the silk (Gauthier, et al., 2004; Asakura, et al., 2006). In addition to the fibroin and sericin proteins, there are other minor components such as small proteins, lipids, and carbohydrates (Gauthier, et al., 2004). The insect cocoon during metamorphosis has been suggested to protect from predators, biodegradation, and dehydration (Gauthier et al, 2004; Zhou et al., 2000).

Cytochrome System of *Bombyx mori*

Once metabolic rates are determined, more advanced questions can be asked, such as - what are the causes of metabolic rate fluctuations? One possible answer could be that there is a higher amount of metabolically active tissue in the organisms with the higher mass-specific $\dot{V}O_2$ as compared to other organisms. One method to determine the activity level of the mitochondria in the tissues is to assay the activity levels of cytochrome oxidase.

Silkworm eggs have a complete set of respiratory chain cytochromes in their mitochondria (Pappenheimer and Williams, 1953; Sanborn and Williams, 1950; Sato and Takesue, 1975; Sato, 1976). The developing larva and pupa of *Hyalophora cecropia* have the cytochrome proteins in place in the electron transport system similar to other organisms (Sato, 1976). Cytochrome c oxidase is a large protein that spans the inner membrane of mitochondria. This enzyme acts as the terminal electron acceptor with elemental oxygen and is described by the formula below:



This enzyme catalyzes the reaction of four hydrogen to two oxygen to produce water. This reaction enables protons to move across the membrane and sets up the chemiosmotic potential across the membrane that activates ATP synthase enzyme to synthesize ATP. The levels of active cytochrome c oxidase will be expected to correlate to changes in the oxygen consumption rates over the life cycle. Mitochondria contain multiple proteins involved in metabolic pathways. The inner membrane of the mitochondria has mostly proteins responsible for oxidative phosphorylation whereas the matrix has the proteins responsible for the citric acid cycle (CAC). Cytochrome c oxidase is the enzyme responsible for catalyzing the final reaction of the electron transport system between cytochrome c and oxygen to produce water – a metabolic byproduct. Approximately 92% of the activity of cytochrome c oxidase occurs in the inner mitochondrial membrane, with the rest occurring in the outer membrane (Ragen et al., 1987). Cytochrome c oxidase makes up about 21% of the mitochondrial proteins (Ragen et al., 1987). The activity of cytochrome c oxidase is typically either measured through absorbance changes or by the rate of oxygen consumption (Ragen et al., 1987). Therefore, the activity level of cytochrome c oxidase can be used as an indirect indicator of metabolic potential.

CHAPTER 2

METABOLIC ALLOMETRY DURING LARVAL DEVELOPMENT AND METAMORPHOSIS OF THE SILKWORM, *BOMBYX MORI*

Introduction

Allometry has been a mainstay of physiological investigation for over a century, with the great majority of studies having focused on the metabolic allometry of adults of various species including the so-called “mouse to elephant curve”. In 1932, Kleiber reported that a proportionality exponent of 0.75 applied for metabolic scaling relationships in mammals. Later, this mass exponent was extended to poikilothermic organisms (Wieser, 1984). Interspecific mass-specific metabolic rate decreases with increasing body mass, and if it follows the quarter power scaling law, it expresses a negative slope of approximately -0.25 on a double logarithmic regression (Couture, 1995; West, 1997). Heusner (1982), however, challenged the 0.75 scaling exponent advocated by Kleiber (1932) and later Wieser (1984), by declaring it a statistical artifact. Heusner (1982) argued that the 0.75 scaling coefficient was created from individual parallel regression lines of 0.67 that followed the Surface Law, but an average scaling coefficient of 0.75 was revealed when these individual regression lines were analyzed together via a regression model. Wieser (1984) stated that the controversy may be due to the lack of distinction between intraspecific and interspecific allometry or between metabolic allometry using whole animal metabolic rate versus mass-specific metabolic rate. Thus, began a long-standing controversy over the 0.75 and 0.66 scaling exponents of metabolic rate as a function of body size in adult animals (Heusner, 1982;

Feldmen and McMahon, 1983; Wieser, 1984; Schmidt-Nielsen, 1984; Smith, 1984; West, et.al., 1997; West et al., 2002; Agutter and Wheatley, 2004; Kozlowski and Konarzewski, 2004; Economo et al., 2005). In general, interspecific poikilotherms scale as expected by Kleiber's law (0.75), however the data are variable between taxa (Agutter and Wheatley, 2004).

Compounding the ongoing uncertainty about metabolic scaling is the extra complexity involving *intra-* vs. *interspecific* scaling relationships, as Wieser (1984) emphasized. Developing animals may not follow general allometric principles and models established for interspecific adult comparisons (Smith, 1984; Burggren, 2005; Hunt Von Herbing, 2006). *Intraspecific* as opposed to *interspecific* allometry has been seldom investigated and an approach that recognizes and controls for both growth (body mass increase) and development (additional differentiation) as determinants of body mass influence on physiological processes should reveal much about not only allometry, but also about development processes.

Insects have important developmental characteristics that make them well suited to allometric studies. For example, insects typically have a brief life cycle as well as highly distinctive morphological markers and discrete stages during their development that make them useful for allometric studies involving growth and development. Insects do have a limitation of a non-expandable exoskeleton that must be shed regularly to allow for increases in size. However, observation of exoskeleton shedding, i.e. ecdysis, can be used by investigators to effectively demarcate developmental stages. Large ranges of body mass are imperative for allometric studies and many insects pass through as many as four orders of magnitude of body mass growth from egg to adult.

There is also a massive morphological reorganization during metamorphosis that occurs that further adds to the complexity of the developing insect. Surprisingly, though, there are relatively few allometric studies published on insects, even though they are the largest, most diverse, and arguably the most complex class of animals.

In this study I investigated *intraspecific* developmental allometry of the silkworm, *Bombyx mori*, by measuring rates of oxygen consumption and carbon dioxide production during successive lifecycle stages from larval instar I through V, prepupal and the pupal stage to determine if metabolic rates during development showed patterns predicted by standard interspecific allometric data based on studies of adult *Bombyx*. The instar I larvae of *Bombyx* weigh approximately 0.0004 g, growing to as much as 2 g in the fifth larval instar before finally decreasing body mass to 0.33 g as short-lived adults. I hypothesized that the changing fat stores, fundamental tissue growth, and remodeling and apoptosis associated with various larval instars, pupation and metamorphosis might result in metabolic allometric patterns that differ from those predicted using standard allometric principles developed from interspecific comparisons.

Materials and Methods

Life History of Bombyx mori

Bombyx mori complete their life cycle in approximately 55 days (Figure 2.1).

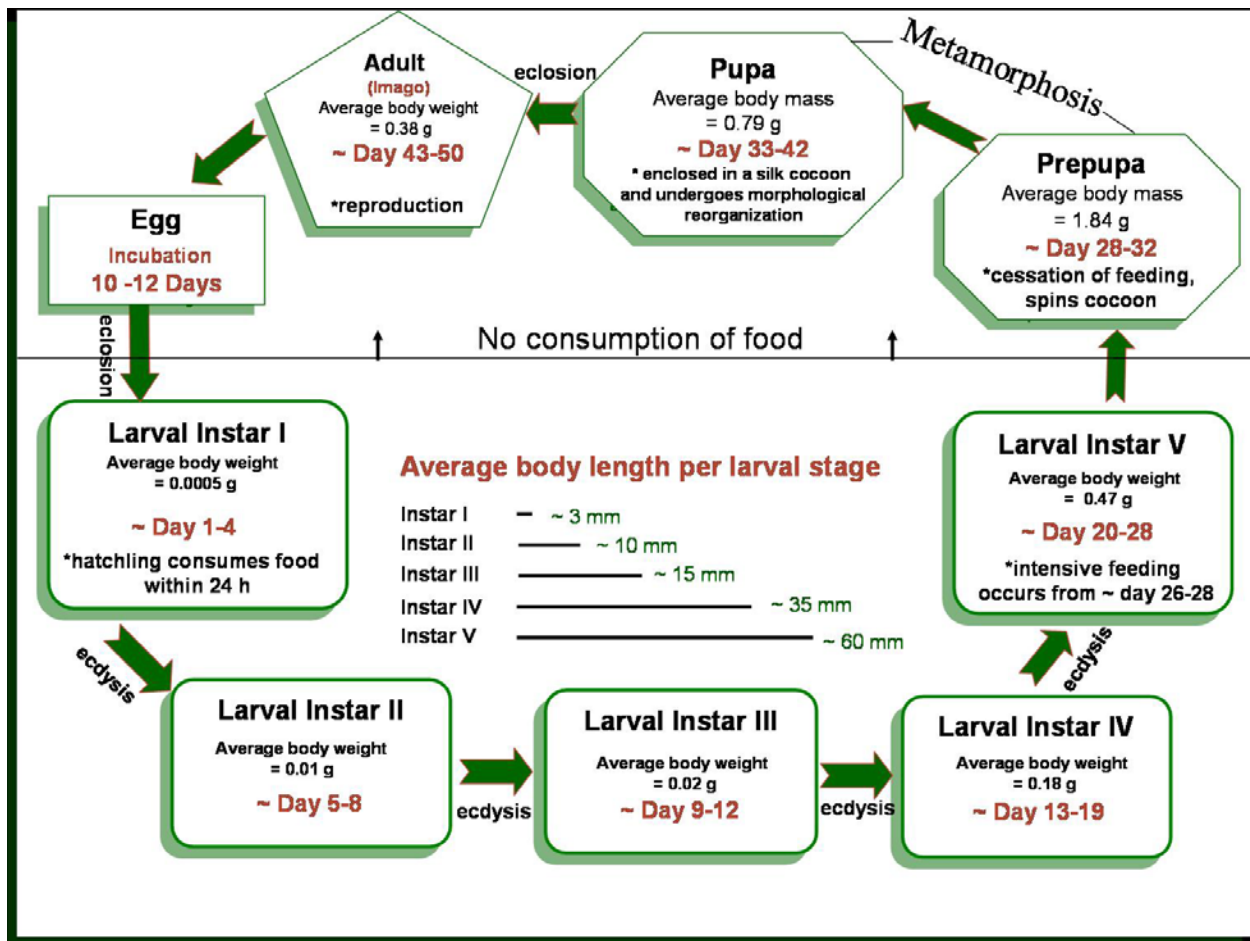


Figure 2.1: Life cycle of *Bombyx mori*.

The incubation period of the developing embryo is approximately 10 days. The instar I larvae, typically 3 mm long and 0.0004 g in body mass, hatch according to circadian rhythms and begin to feed within 24 hours. There are five successive instars that are behaviorally and morphologically separated by ecdysis (molting). During the prepupal stage, the larva (now approximately 2 g body mass) prepares for metamorphosis by constant feeding. The food consumed during this phase serves as the energy storage for the remainder of the life cycle as the short-lived adults do not possess functional mouth parts. At the end of the prepupal stage, the larva searches for an appropriate place to spin a cocoon of silk around its body. Once the cocoon is

formed, the larva secretes a hard outer cuticle and metamorphosis commences. Metamorphosis is a complex process that includes hyperplastic and hypertrophic growth as well as apoptosis and histolysis of certain larval tissues. After about ten days, the adult emerges out of the cocoon, i.e. eclosion, and lives for approximately five days, during which time mating and egg laying occur.

Animal Husbandry

Fertilized eggs of the silkworm, *Bombyx mori*, were obtained from Carolina Biological Supply, Mulberry Farms and Coastal Silkworms. Upon arrival in the laboratory, the eggs were placed in Petri dishes and incubated in a transparent incubator at a temperature of 25 ± 0.5 °C with a relative humidity of $60 \pm 5\%$. The incubators were illuminated with a LD 12:12 photoperiod. The Petri dishes were examined for hatched larvae each morning, since hatching, i.e. eclosion, occurs in the morning due to circadian rhythms of metabolism and eclosion hormone secretion (Fugo et. al, 1984). Newly hatched instar I larvae were transferred into date-labeled Petri dishes. All larval instars were fed an artificial silkworm diet obtained from Carolina Biological Supply. Food remaining in the Petri dishes was removed daily in the evening.

Respirometry System

Many investigations of metabolic allometry have measured oxygen consumption ($\dot{V}O_2$), an accepted indirect method of estimating aerobic metabolic rate (McMahon, 1973; Else and Hubert, 1981; German, 1989; West, et. al, 1997; West, 2002; Kozlowski et al., 2003; Brown et al., 2004; Economo, 2005, and others). Closed system respirometry was utilized in this study to measure both $\dot{V}O_2$ and carbon dioxide production ($\dot{V}CO_2$) during the entire life-cycle of *Bombyx mori*. Oxygen consumption

caused a fall in pO_2 of approximately 2 mmHg to no more than 10.0 mmHg during measurements, for that reason the increase in pCO_2 inside of the respirometer was considered have a negligible affect. The larvae and pupae were placed in glass syringes, varying in Volume depending on larval size (Table 1). A 20-gauge needle was fastened securely onto the syringe and inserted into a rubber stopper, thus creating a watertight, gas-tight closed respirometer of known Volume (Figure 2.2). All measurements were made at 25 ± 0.5 °C.

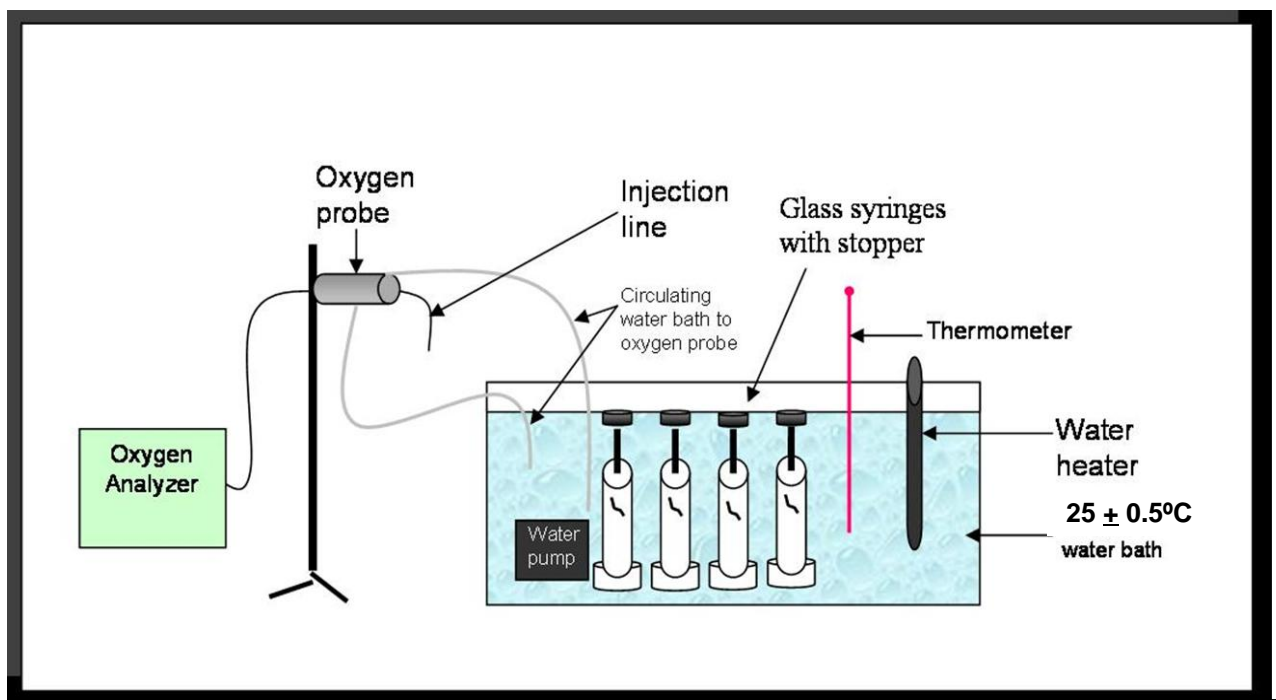


Figure 2.2: Oxygen consumption schematic diagram.

Measurements of $\dot{V}O_2$ and $\dot{V}CO_2$ were performed daily on larvae and pupae pre-weighed to the nearest milligram. All of the holding chambers, i.e. syringes, water bath, incubator, were transparent and measurements were made during daylight hours to prevent disruption of normal circadian rhythms. Hormonal activity has been linked to the light-dark cycle in silkworms (Fugo, 1984), and changes in light and / or dark has also

been shown to alter the $\dot{V}O_2$, heart rate and / or cause heart rate reversals in other insect species (Stusek, 2000; Kazuyuki et. al, 2005). All measurements were made approximately 12 hours after feeding to avoid specific dynamic action increases in $\dot{V}O_2$.

Random larvae were selected for measurements each day from a population ranging from approximately 500 to 1,000 larvae. The larvae were retrieved from the incubator and placed in a gas-tight glass syringe for 30 min before a baseline $\dot{V}O_2$ or $\dot{V}CO_2$ measurement was made to ensure that larvae were in a quiescent state and maintaining a standard metabolic rate before measurements were taken. Larvae were periodically viewed during the holding and measurement period to confirm their inactivity. Any active larvae were removed from the experiment. Individual larvae were measured three times each day and mean $\dot{V}O_2$ or $\dot{V}CO_2$ was calculated for each larvae. The syringe was refreshed with air at $25 \pm 0.5^\circ C$ between each reading. A measured Volume of air (~0.5 ml) was injected directly from the syringe containing the animal into the oxygen or carbon dioxide analyzer at specified time periods, as indicated for various life stages (Table 2.1).

Life Cycle Stage	Total elapsed time in syringe (min)	Initial syringe Volume (minus body mass) (ml)
Larval Instar I (~0.0004 - 0.0009g)	180	0.5
Larval Instar II (~0.0009-0.015g)	150	0.5
Larval Instar III (~0.015 – 0.250g)	90	0.5 – 10
Larval Instar IV (~0.250 – 0.750g)	30	10 -20
Larval Instar V (~0.750 – 1.5 g)	15	20 - 30
Pre-Pupa (~ 1.50 – 3.0g)	30	50
Pupa (~0.70 – 2.0 g)	30	30

Table 2.1: Respirometry variables for oxygen consumption measurements.

Oxygen Consumption Measurements and Calculations

Oxygen levels in 0.5 ml gas samples injected directly from the syringe respirometers were measured with a thermostatted Clark-type oxygen electrode (Radiometer, Model PHM 72 Mk-2). Each day, barometric pressure was recorded and pO_2 of ambient air was calculated by subtracting water vapor pressure. The electrode was calibrated with a two-point calibration using atmospheric air and pure nitrogen prior to each experiment and after each measurement period throughout the day.

Oxygen consumption was calculated using the formula:

$$\dot{V}O_2 = \frac{([O_2]_{t1} - [O_2]_{t2}) * V \text{ (ml)}}{T \text{ (h)}}$$

where $t1$ = initial time, $t2$ = end time, V = Volume of syringe minus animal mass.

Mass-specific oxygen consumption was calculated using the formula:

$$\dot{V}O_2 = \frac{([O_2]_{t1} - [O_2]_{t2}) * V \text{ (ml)}}{T \text{ (h)} * BM \text{ (g)}}$$

where BM = body mass.

$\dot{V}O_2$ rates are reported as whole animal ($ml\ O_2 \cdot hr^{-1}$) and mass-specific ($ml\ O_2 \cdot g^{-1} \cdot hr^{-1}$). Mean \pm 1 standard error (s.e.) was calculated for body mass and mass-specific $\dot{V}O_2$ as a function of developmental stage.

For determining gender-based $\dot{V}O_2$ differences, newly hatched instar I larvae were placed into separate, labeled Petri dishes for the remainder of their life cycle. $\dot{V}O_2$ measurements were made throughout each instar as well as the pupal stage using new larvae each day. Larvae during instar V and pupae were examined and morphological characteristics were recorded. When the adults emerged, gender was confirmed. There were no discrepancies found in gender determination between these stages. Some of the silkworms died before gender could be determined and data from these insects

were not included with the gender data. The adult males were not separated in from the females in this study.

Carbon Dioxide Production Measurements and Calculations

$\dot{V}\text{CO}_2$ was measured for each instar using the respirometry techniques described above. Random larvae were selected from a population of approximately 500 to 1,000 silkworms that were the median age of each developmental stage. Approximately 0.5 ml of gas from the syringe was injected into a Beckman Medical Gas Analyzer (Model LB-2). The combinations of syringe Volume, elapsed time, gas Volume and gas injected from the syringe for each animal are provided in Table 1.

Carbon dioxide production ($\text{ml CO}_2 \text{ hr}^{-1}$ and $\text{ml CO}_2 \text{ g}^{-1}\text{hr}^{-1}$) was calculated using the following formula:

$$\dot{V}\text{CO}_2 = \frac{([\text{CO}_2]_{t1} - [\text{CO}_2]_{t2}) * V (\text{ml})}{T(\text{h}) * \text{BM} (\text{g})}$$

Mean \pm 1 s.e. was calculated for each developmental stage.

Heart Rate Measurements

The heart cannot be viewed through the dorsal cuticle during larval instars I, II and III, therefore a pilot study utilizing an impedance converter was implemented with larval instar IV and V for comparison to the manual heart rate counts. Copper wires (40 gauge) were inserted under the cuticle of larval instar IV and V animals at the 5th body segment. The wires were affixed with instant adhesive to the cuticle and the larva was placed into a Petri dish. The wires were connected to an impedance converter (UFI model 2991) that was connected to a Power Lab system using Chart software. Cardiac contractions were recorded for a period of approximately 2 h.

Heart rates during instar IV and V was manually counted by viewing the heart through the dorsal epithelium.

Analyses and Statistics

Each instar for $\dot{V}O_2$ was analyzed using simple non-linear regression models on semi-logarithmic plots. The graphs were subsequently transformed to double logarithmic axes to obtain a linear relationship. Simple linear regressions were performed on the data sets (both whole animal and mass-specific $\dot{V}O_2$) to determine scaling coefficients. The selected alpha level for statistical decisions was $\alpha = 0.05$. A Shapiro-Wilkes normality test determined the normality of the data. A one-way ANOVA determined if the means were different among each lifecycle stage for both whole animal and mass-specific $\dot{V}O_2$. A Student Neuman-Keuls (SNK) was utilized to compare all pair-wise combination of means. Slopes of each instar were compared via t distribution to the overall slope during development. A simple linear regression was performed on the mean values of $\dot{V}O_2$ as a function of body mass and the slopes were calculated. T-tests were calculated for comparison of slopes of the mean values of $\dot{V}O_2$ as a function of body mass by instar to the overall slope during development. An independent t-test was performed on each instar between genders to determine if there was a significant difference in either mean whole organism or mass-specific $\dot{V}O_2$.

A simple linear regression was performed on the $\dot{V}CO_2$ data on double logarithmic axes to determine slopes. A one-way ANOVA was performed on the $\dot{V}CO_2$ data by stage and an SNK was utilized to compare all pair-wise combination of means.

Respiratory quotients were calculated for each instar by dividing the mean $\dot{V}CO_2$ by the mean $\dot{V}O_2$ for each developmental stage.

Results

Body Mass Changes

Body mass of *Bombyx mori* increased progressively from a mean and s.e. of 0.00045 ± 0.00024 g in the first instar to 1.84 ± 0.10 g in the prepupal stage. From this peak, body mass decreased to a mean and s.e. of 0.79 ± 0.30 g in the pupal stage and decreased further to 0.38 ± 0.03 g in the adults (Figure 2.3).

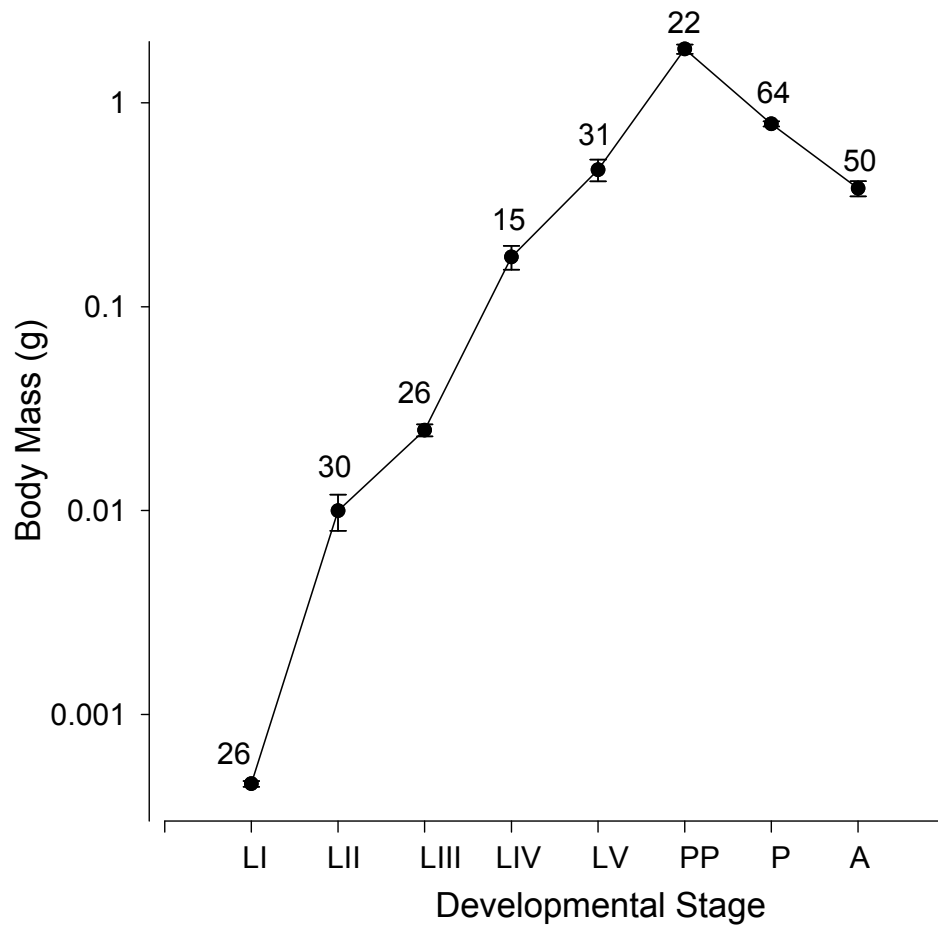


Figure 2.3: Body mass of developing *Bombyx mori* as a function of developmental stage. Mean \pm s.e., and n are shown.

Oxygen Consumption

Whole animal oxygen consumption

A regression analysis on a semi-logarithmic plot showed significant relationships between $\dot{V}O_2$ and body mass for instar II to prepupal stages (Figure 2.4A). Larval instar II through the prepupal stage showed significant relationships ($r^2_{adj} = 0.92, 0.76, 0.78, 0.93, 0.69$). Average $\dot{V}O_2$ increased significantly ($p < 0.001$) from a mean and s.e. of 0.00064 ± 0.000047 ml $O_2 \cdot hr^{-1}$ in instars I to 0.77 ± 0.06 ml $O_2 \cdot hr^{-1}$ in the prepupal stage and then decreased to 0.21 ± 0.01 ml $O_2 \cdot hr^{-1}$ in the pupal stage (Figure 2.3B). $\dot{V}O_2$ in adult females increased to a mean of 0.35 ± 0.03 ml $O_2 \cdot hr^{-1}$. A Student-Newman-Keuls analysis separated these means into six statistically significant groups: A: larval instar I; B: larval instar II; C: larval instar III; D: larval instar IV; E: larval instar V and pupae; F: prepupae; G: adults.

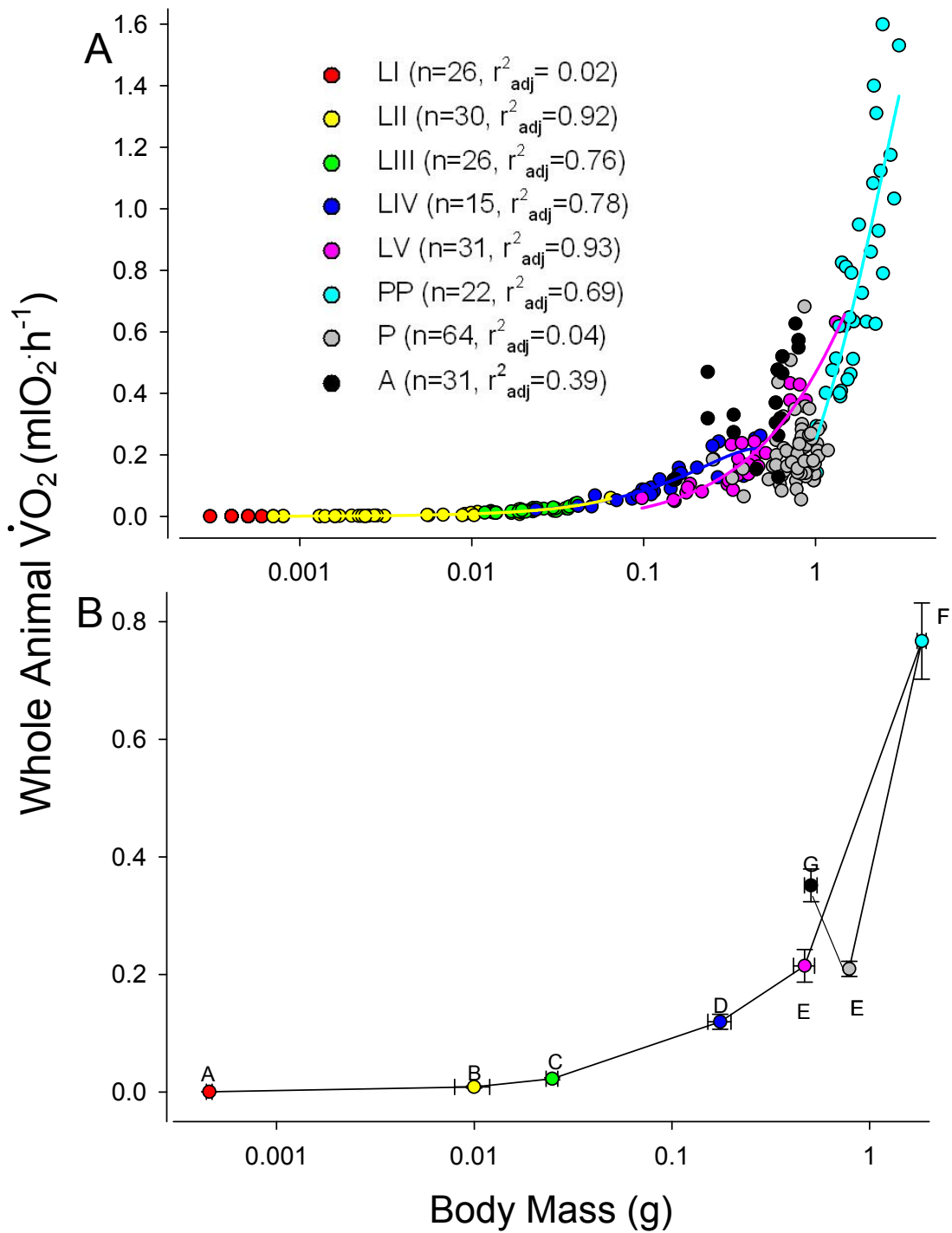


Figure 2.4: Semi-log plots of (A) whole animal $\dot{V}O_2$ of developing *Bombyx mori* as a function of body mass and (B) mean whole animal $\dot{V}O_2$ as a function of mean body mass. Significant simple polynomial regression lines, regression coefficients, mean ± 1 s.e., SNK groups are shown by unique letters, and n are shown.

$\dot{V}O_2$ was not correlated with body mass in larval instar I ($r^2_{adj} = -0.02$; t-test for slopes, $p = 0.57$) (Figure 2.5A). $\dot{V}O_2$ and body mass during larval instars II, III, IV and V all showed significant ($p < 0.0001$) relationships ($r^2_{adj} = 0.93, 0.73, 0.96, 0.89$) and had similar slopes ($b = 0.99, 0.96, 1.00, \text{ and } 1.02$). The prepupal stage had a significant ($p = 0.001$) relationship ($r^2_{adj} = 0.66$) with a slope of 1.49. The pupal stage did not depict an overall relationship ($r^2_{adj} = 0.04$) and had a slope of 0.38 that was not significantly different than zero ($p = 0.06$).

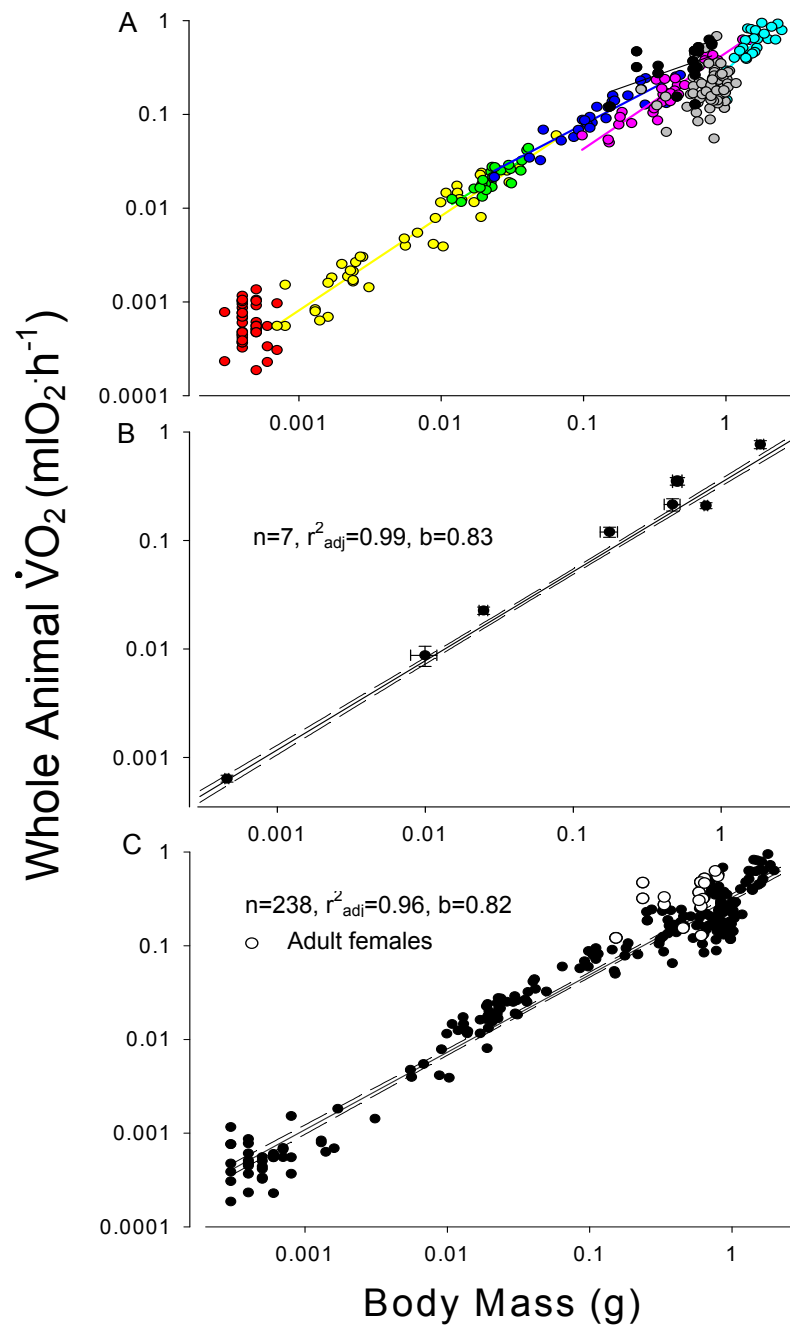


Figure 2.5: Double log plots of (A) whole animal $\dot{V}O_2$ as a function of body mass in developing *Bombyx mori*, (B) mean whole animal $\dot{V}O_2$ by developmental stage as a function of body mass, and (C) whole animal $\dot{V}O_2$ as a function of body mass throughout development. Significant simple linear regressions with adjusted regression coefficients, 95% confidence intervals, n values and slopes are shown for each developmental stage and throughout development.

T-tests for slopes showed that larval instar I, III, V and the pupal stage were not significantly different from the overall slope of 0.82 during development. Larval instar II ($0.005 > p > 0.002$), instar IV ($p < 0.001$), and the prepupal stage ($p < 0.001$) slopes were significantly different from the overall slope during development of 0.82 (Figure 2.5A). Using the mean values for $\dot{V}O_2$ for each stage, the overall slope for $\dot{V}O_2$ as a function of development was 0.83 ($r^2_{adj} = 0.99$, $p < 0.0001$) (Figure 2.5B). Finally, all $\dot{V}O_2$ data were plotted irrespective of developmental stage and coefficient of determination and slope determined as $r^2 = 0.96$ and $b = 0.82$ ($p < 0.0001$) (Figure 2.5C).

To further determine the most appropriate method for data presentation, a t-test for slopes revealed no significant difference between the slope of 0.83 for mean whole animal $\dot{V}O_2$ (Figure 2.5B) and 0.82 for whole animal $\dot{V}O_2$ throughout development (Figure 2.5C).

Mass-specific oxygen consumption.

Regression analyses on a semi-logarithmic plot showed no significant relationships between mass-specific $\dot{V}O_2$ and body mass throughout development (Figure 2.6A). Mean mass-specific $\dot{V}O_2$ decreased approximately six orders of magnitude over development from a mean and s.e. of $1.38 \pm 0.12 \text{ ml O}_2 \cdot \text{g}^{-1} \text{hr}^{-1}$ in the first instar to $0.28 \pm 0.02 \text{ ml O}_2 \cdot \text{g}^{-1} \text{hr}^{-1}$ in the pupal stage (Figure 2.6B) before rising again in the adult. $\dot{V}O_2$ variability within each instar also decreased over development.

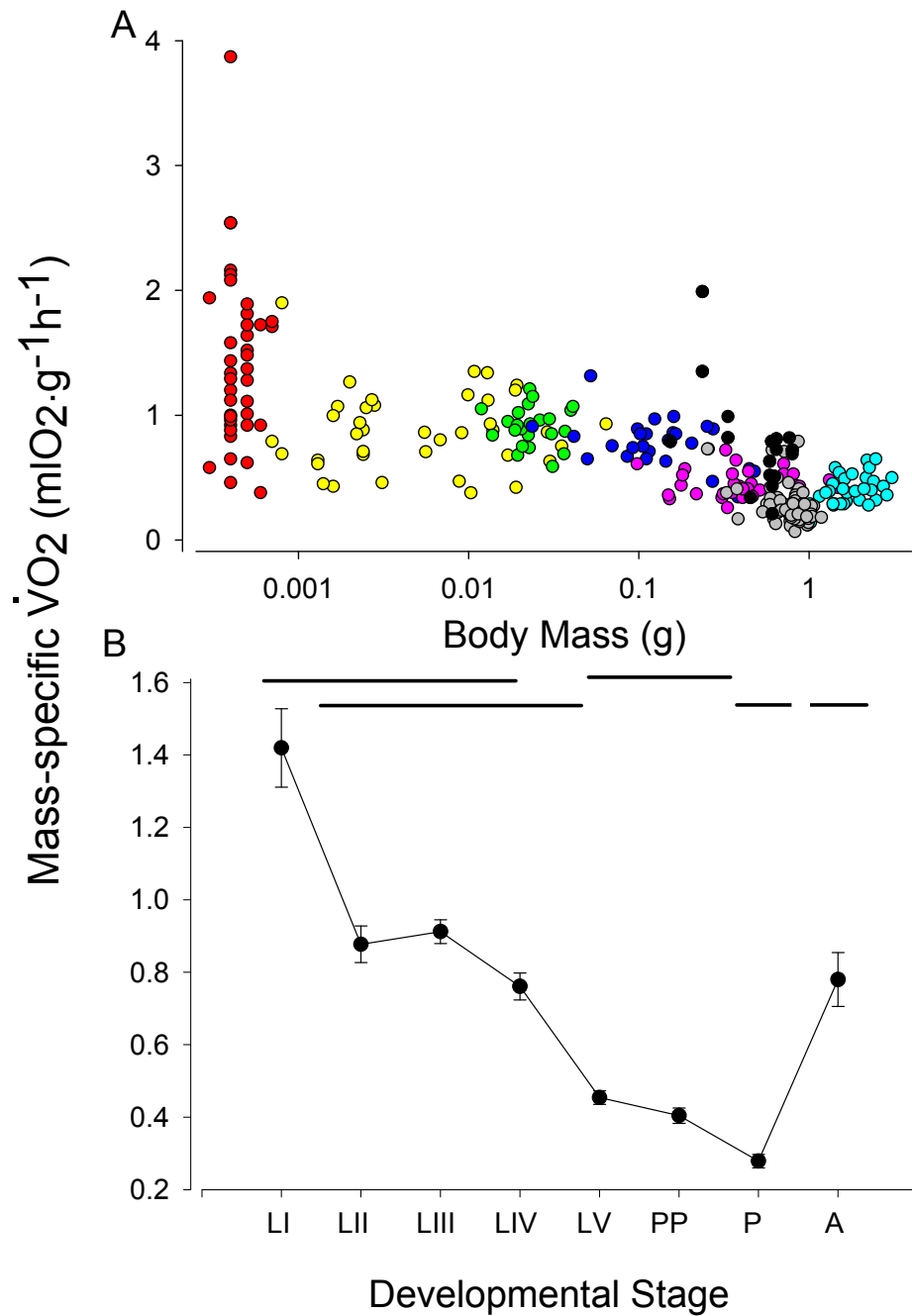


Figure 2.6: (A) Semi log plot of mass-specific $\dot{V}O_2$ as a function of body mass of developing *Bombyx mori* and (B) mean mass-specific oxygen consumption as a function of developmental stages of *Bombyx mori*. Mean \pm one s.e., SNK groups (horizontal lines), and n values are shown.

A SNK separated the means into five statistically significant groups ($p < 0.001$): A: larval instar I, larval instar II, larval instar III; B: larval instar II, larval instar III, larval instar IV; C: larval instar V, prepupae; D: pupae; E: adults (Figure 2.6B).

To determine the slope for each developmental stage of mass-specific $\dot{V}O_2$, data were plotted on double logarithmic axes to achieve a linear relationship (Figure 2.7A). There were no significant relationships found by simple linear regression analysis for any of the individual developmental stages. A simple linear regression analysis on mean mass-specific $\dot{V}O_2$ for each developmental stage as a function of body mass (Figure 2.7B) yielded an $r^2 = 0.54$ and a slope of -0.17.

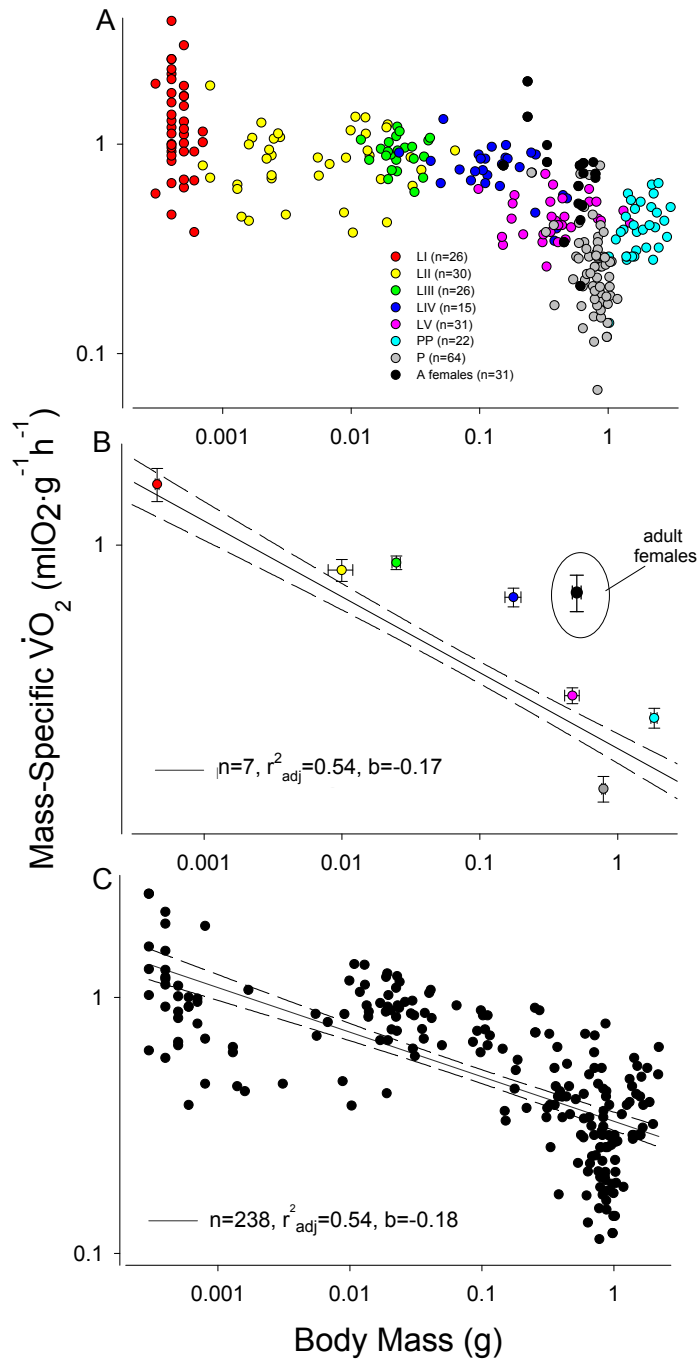


Figure 2.7: (A) Double log plot of mass-specific $\dot{V}O_2$ as a function of body mass across development of *Bombyx mori*. (B) Mean mass-specific $\dot{V}O_2$ as a function of mean body mass of developing *Bombyx mori*. (C) Mass-specific $\dot{V}O_2$ as a function of body mass across development of *Bombyx mori*. Significant simple linear regression with adjusted regression coefficients, 95% confidence intervals, n values and slope values are shown for each developmental stage.

Finally, all mass-specific $\dot{V}O_2$ data were plotted irrespective of developmental stage and the coefficient of determination and slope determined as $r^2 = 0.54$ and $b = -0.18$ ($P < 0.0001$) (Figure 2.7C).

To further determine the appropriate method for data presentation, a t-test for slopes was performed to determine differences between mass-specific whole animal $\dot{V}O_2$ (Figure 2.7B) and whole animal $\dot{V}O_2$ throughout development (Figure 2.7C) and no significant difference was found between the slopes of -0.17 and -0.18 ($p > 0.10$).

Effect of Gender on $\dot{V}O_2$

None of the larval instars showed significant differences in either whole animal or mass-specific $\dot{V}O_2$ (Figure 2.8). However, prepupal females had significantly higher whole animal mean $\dot{V}O_2$ than prepupal males ($p = 0.031$), and whole animal and mass-specific $\dot{V}O_2$ of adult males was significantly higher than in females ($p < 0.02$). Notably, prepupal females were significantly larger in body mass than the males of the same age ($p < 0.0001$).

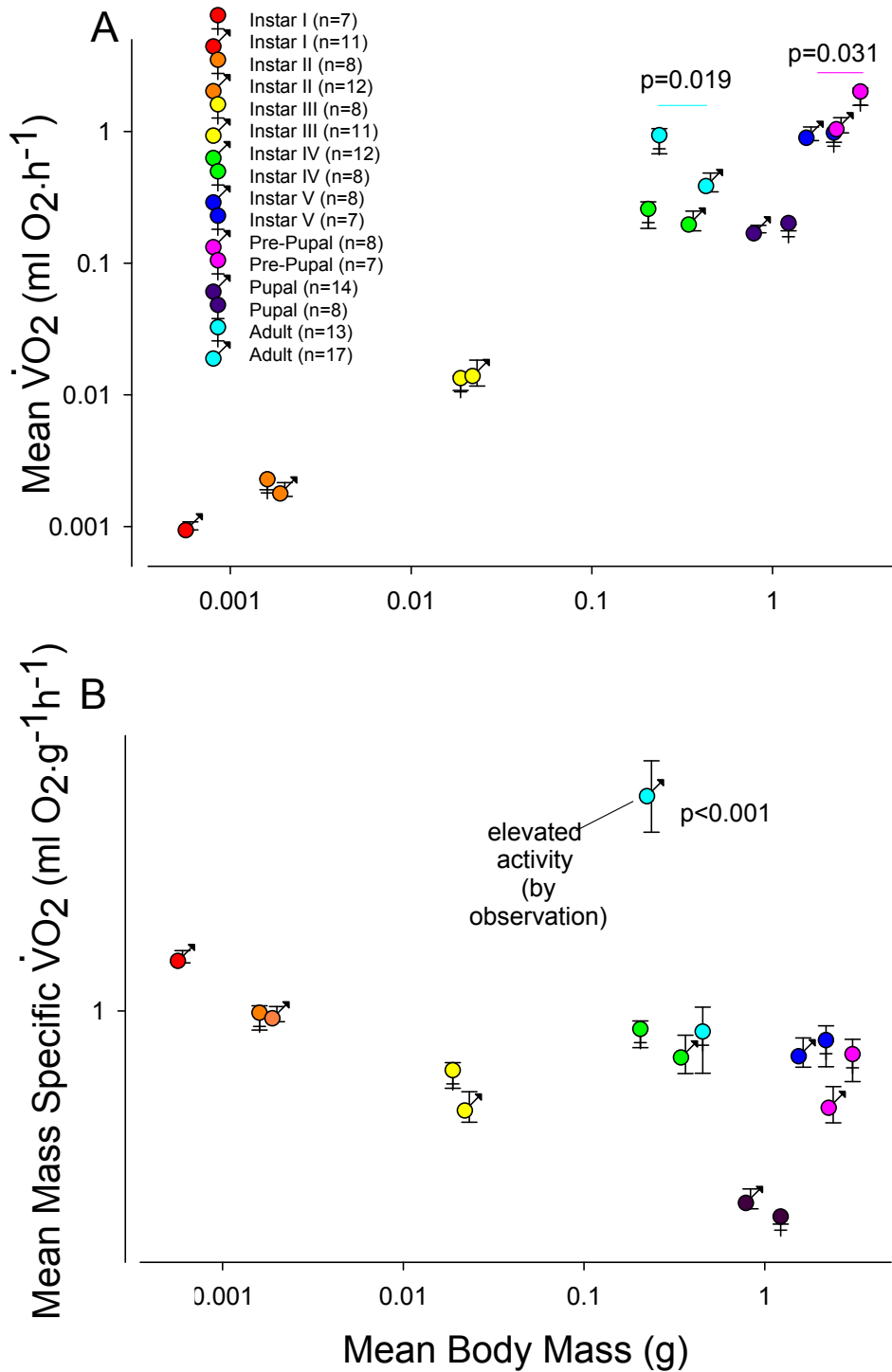


Figure 2.8: Double log plot of mean (A) whole organism and (B) mass-specific oxygen consumption as a function of body mass by gender in developing *Bombyx mori*. Standard error is shown. All data male-female data pairs are not significantly different from each other, with the exception of the male-female pair for adults, where the male is significantly higher than female ($P < 0.001$).

$\dot{V}O_2$ and Surface Area

Whole animal $\dot{V}O_2$ as a function of total surface area (Chapter 4) was plotted on double logarithmic axes (Figure 2.9A) and the r^2 was 0.85 and the slope was 0.77 ($p < 0.0001$). The mass-specific $\dot{V}O_2$, when plotted on double logarithmic axes (Figure 2.9B), revealed an r^2 value of 0.47 and a slope of -0.10 and was not significantly different than zero ($p = 0.13$).

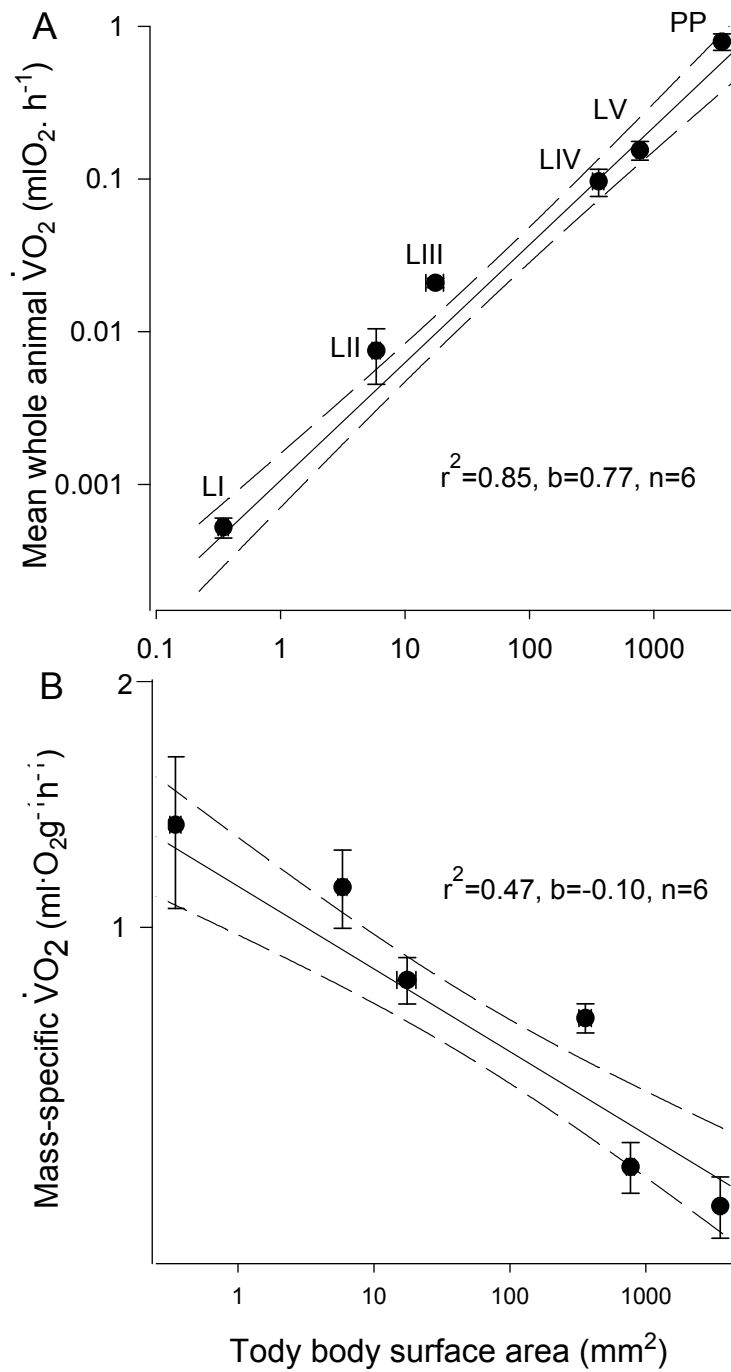


Figure 2.9: Double log plot of mean (A) whole animal $\dot{V}O_2$ as a function of total surface area of *Bombyx mori*. (B) mass-specific $\dot{V}O_2$ as a function of total surface area of developing *Bombyx mori*. Simple linear regression with 95% confidence intervals, n values and slope values are shown for each developmental stage.

Carbon Dioxide Production

Whole animal $\dot{V}\text{CO}_2$ increased significantly (one-way ANOVA, $p < 0.001$) over development with a decrease in the pupal stage (Figure 2.10A). Mean whole animal $\dot{V}\text{CO}_2$ ranged from $0.01 \pm 0.0006 \text{ ml O}_2 \cdot \text{hr}^{-1}$ in larval instar I to $0.62 \pm 0.07 \text{ ml O}_2 \cdot \text{hr}^{-1}$ in the prepupal stage. An SNK separated the means into two statistically significant groups: A: larval instar I, larval instar II, larval instar III, larval instar IV; B: larval instar III, larval Instar IV, larval Instar V, prepupae, pupae, adults.

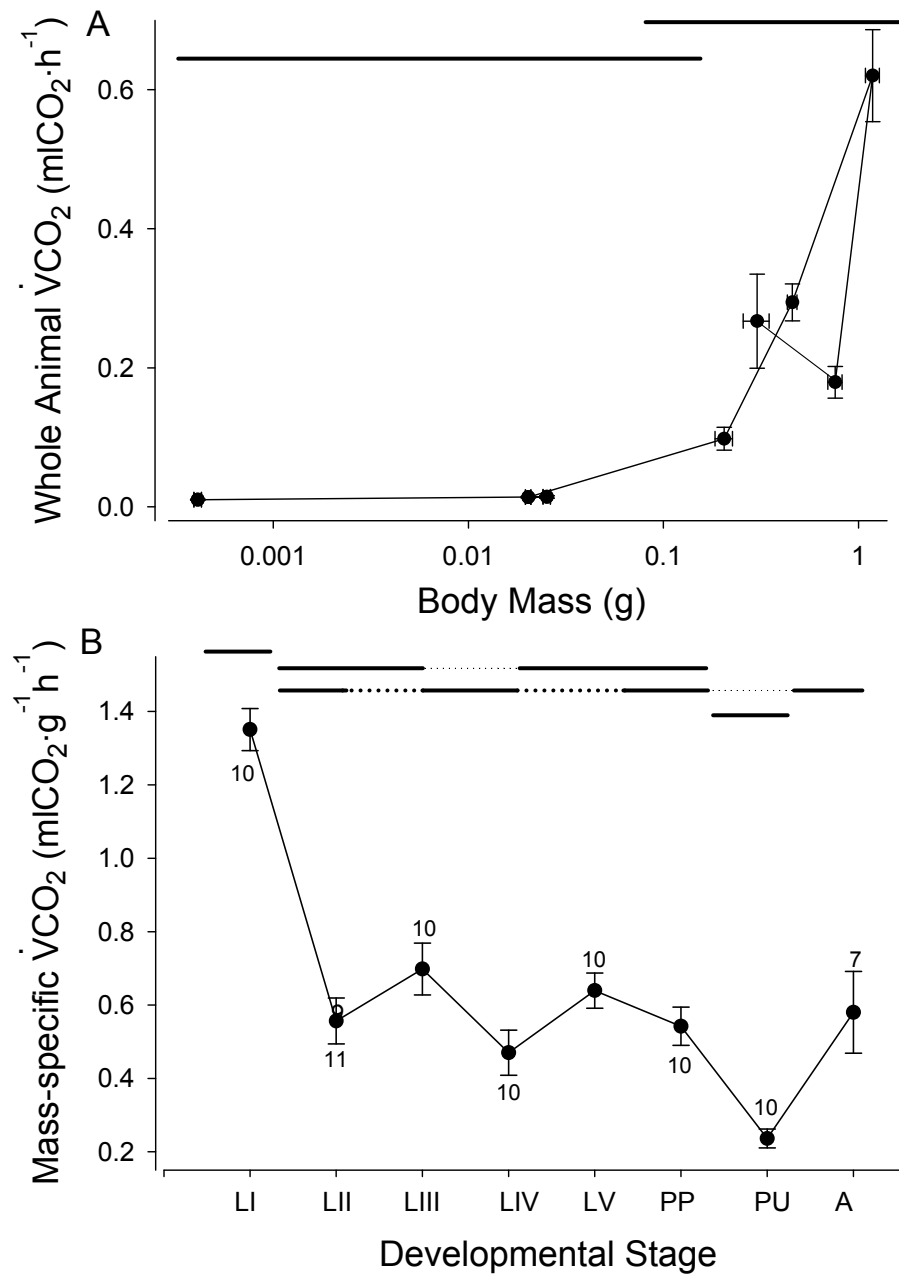


Figure 2.10: (A) Whole animal $\dot{V}CO_2$ as a function of body mass of developing *Bombyx mori* and (B) mass-specific $\dot{V}CO_2$ by developmental stage. Mean, s.e., SNK groups (horizontal lines) and n values are shown.

Mean mass-specific $\dot{V}CO_2$ decreased significantly ($p < 0.001$) over development (Figure 2.10B), ranging from 1.35 ± 0.06 ml O₂·g⁻¹·hr⁻¹ in the LI stage down to $0.24 \pm$

0.03 ml O₂·g⁻¹·hr⁻¹ in pupae. An SNK separated the means into four statistically significant groups: A: larval instar I; B: larval instar II, larval instar III, larval instar V, prepupae; C: larval instar II, larval instar IV, prepupae, adults; D: pupae.

$\dot{V}CO_2$ plotted as a function of body mass on a double logarithmic graph yielded an r^2 value of 0.76 and a slope of 0.52 ($p < 0.0001$) (Figure 2.11A). The mass-specific $\dot{V}CO_2$, plotted on a double logarithmic plot (Figure 2.11B), revealed an r^2 value of 0.46 and a slope of -0.14 ($p < 0.0001$).

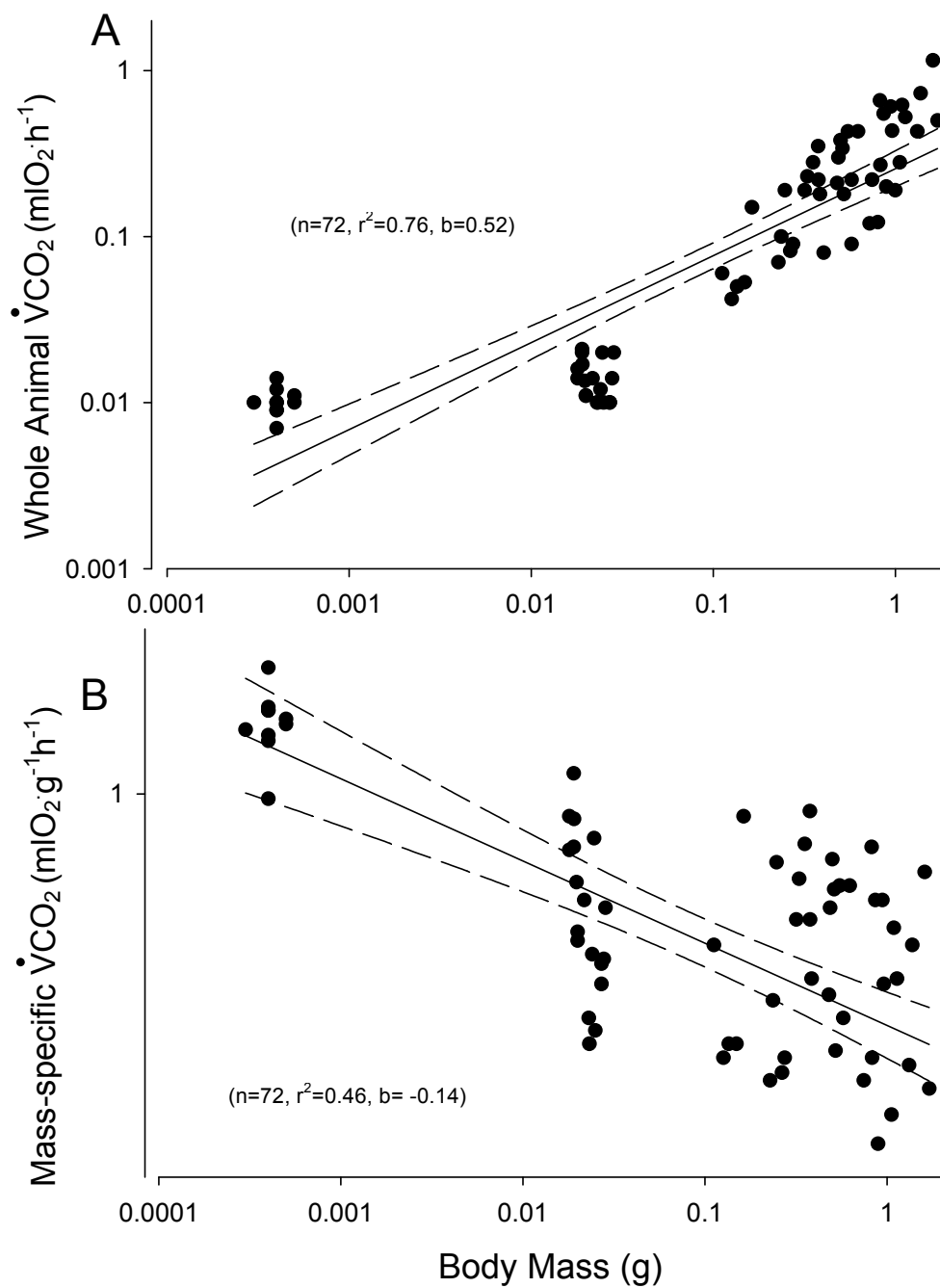


Figure 2.11: Double log plot of (A) whole organism and (B) mass-specific carbon dioxide production as a function of body mass in developing *Bombyx mori*. Simple linear regression with 95% confidence intervals, n values, regression coefficients and slopes are shown.

Respiratory Quotient

Larval instar I had an RQ of 1.10 (Figure 2.12A), instar II had an RQ of 0.57, instar III had an RQ of 0.77 and instar IV had an RQ of 0.60. Larval instar V and the prepupal stage both had an increase in RQ of 1.42 and 1.59, pupae had an RQ of 0.86, and adults had an RQ of 0.74 (Figure 2.12B).

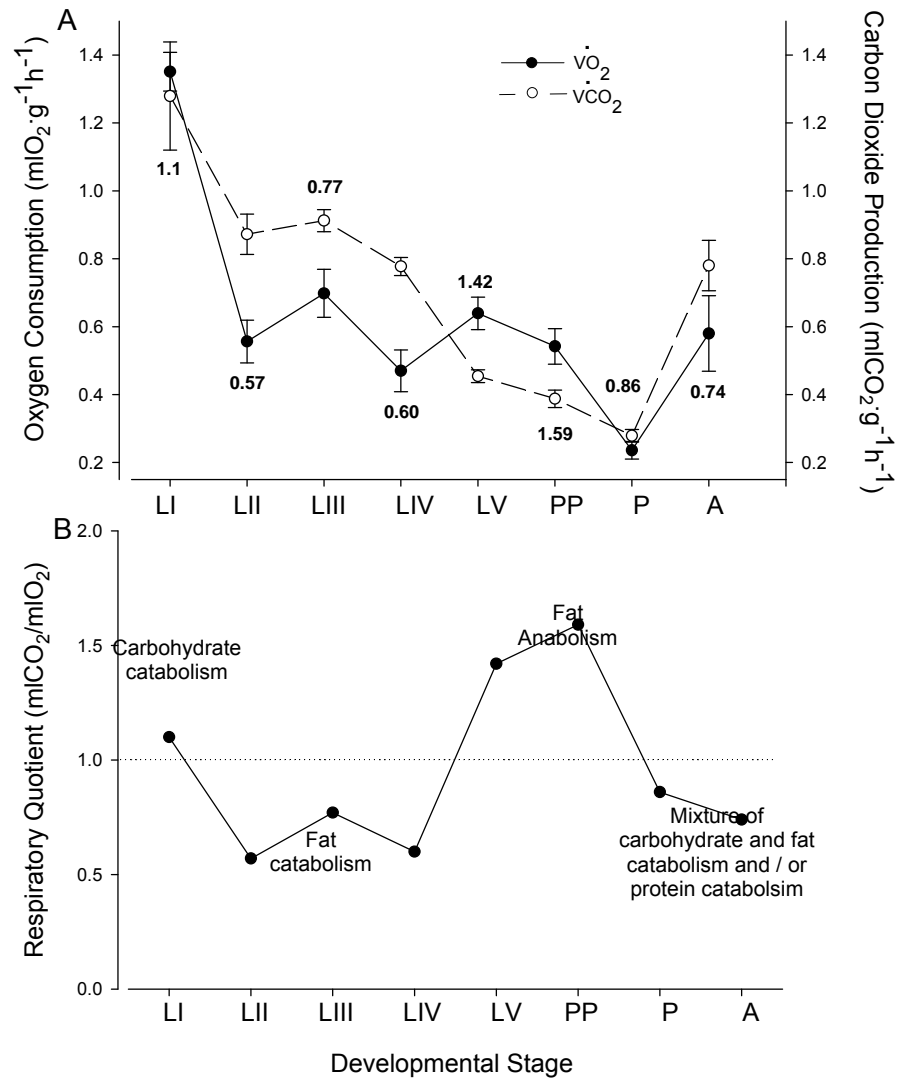


Figure 2.12: (A) Mass-specific $\dot{V}O_2$ and $\dot{V}CO_2$ as a function of developmental stage of *Bombyx mori* and (B) respiratory quotient values as a function of developmental stage. Mean ± 1 s.e, and RQ values are shown. Possible causes for RQ values are shown.

Heart Rate

Heart rate for larval instar IV was 44.0 ± 1.6 bpm and larval instar V was 47.0 ± 1.1 bpm (Figure 2.13). An independent t-test showed no significant difference between instars ($p=0.22$), and a non-linear regression analysis showed no significant relationship between body mass and heart rate ($r^2=0.06$, $p>0.10$).

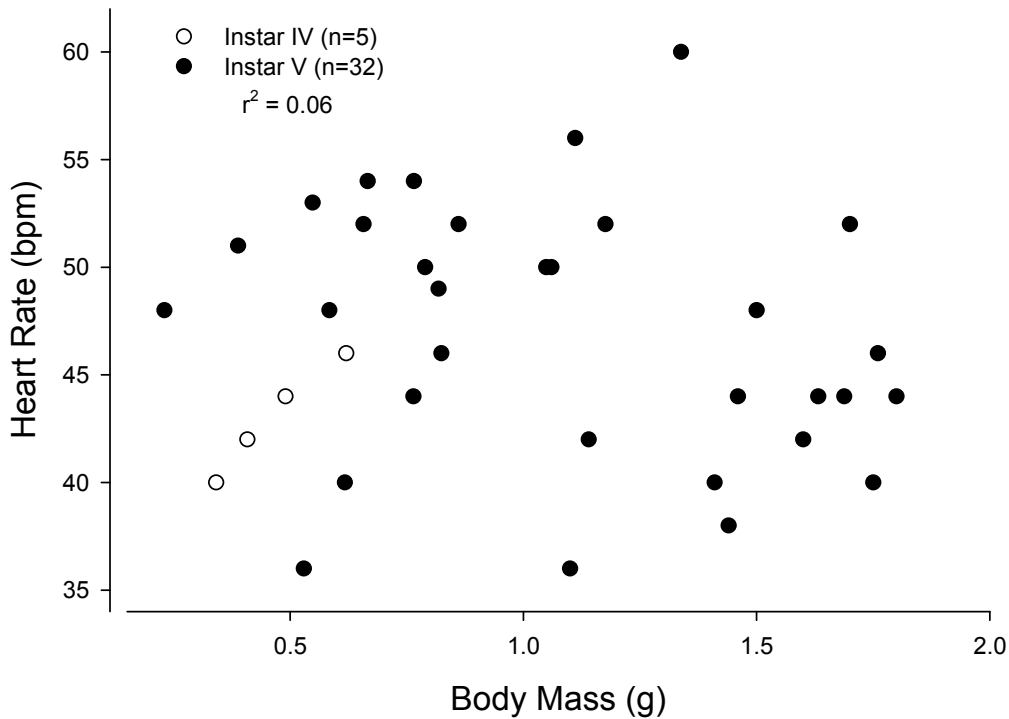


Figure 2.13: Heart rate as a function of body mass. Polynomial simple linear regression is shown.

Discussion

Body Mass and Development

Bombyx mori showed changes in body mass during its life cycle, routinely gained body mass through earlier larval stages and reached maximum weight during the prepupal stage. Within an instar, arthropod body mass can double and this increase in mass does not correspond with an increase in the respiratory system until the cuticle is

shed during ecdysis (Greenlee and Harrison, 2004). This increase in mass without a coinciding increase of the tracheal system can influence respiration, i.e. ventilation rates (Greenlee and Harrison, 2004). This, in turn, might cause an overall decrease in pO_2 of tissues and could contribute to the initiation of ecdysis (Greenlee and Harrison, 2004). After body mass sharply increases through all larval instars, body mass declined starting with the prepupal stage. In some holometabolous insects, i.e. silkworms, beetles, flies, etc., the prepupal stage will be the last stage to eat because the adults lack functional mouth parts and only exist briefly to reproduce (Rockstein, 1973; Ganga, 2003). Once these insects enter metamorphosis, the energy stored during the prepupal stage will be the sole source available for the remainder of the lifecycle. Not surprisingly, then, once the insect reaches a maximum weight in the prepupal stage, the body mass decreases with age during a brief adulthood.

$\dot{V}O_2$ Comparison with Other Insects

Mean whole animal oxygen consumption ranged from $0.0005 \text{ ml O}_2 \cdot \text{h}^{-1}$ in instar I to $0.77 \text{ ml O}_2 \cdot \text{h}^{-1}$ in the prepupa. This compares favorably with data on other insects such as the adult cricket (*Hophloshyrum griseus*) at $0.017 \text{ ml O}_2 \cdot \text{h}^{-1}$ and the woollybear caterpillar larvae (*Gynaephora groenlandica*) at $\sim 0.23 \text{ ml O}_2 \cdot \text{h}^{-1}$ (Bennett, et al., 1999; Nespolo et al., 2003). Mass-specific oxygen consumption of *Bombyx mori* ranged from a mean of $1.38 \text{ mlO}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ in larval I to $0.77 \text{ ml O}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ in the pupa. These are similar to other insects such as the codling moth larva (*Carpocapsa saltitans*) at $0.68 \text{ mlO}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ (Tenney, 1985), and the wintering bee (*Megachile rotundata*) at $0.70 \text{ mlO}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ to $1.90 \text{ mlO}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ (Kemp, 2004).

$\dot{V}O_2$ Allometry

The general intraspecific pattern of change of $\dot{V}O_2$ during development in *Bombyx mori* is generally similar with that predicted by interspecific comparisons of adult organisms, e.g. the “mouse to elephant curve”. However, interspecific allometry does not accurately reflect the pattern of $\dot{V}O_2$ change evident *within* individual instars of silkworms, where b values ranged from 0.96 to 1.49 (Figure 2.5A). The relationship between mass-specific $\dot{V}O_2$ and body mass also differed from instar to instar. Rather than showing a negative slope for the relationship between mass-specific $\dot{V}O_2$ and body mass, during larval instar II and prepupa, mass-specific $\dot{V}O_2$ actually increased slightly as body mass increased. Such changes are contrary to interspecific allometric predictions. A rise in oxygen consumption during molting has been reported for other insects (Edwards, 1953; Rockstein, 1973). This positive relationship might be attributed to the oldest and consequently, the largest larvae within each stage undergoing the process of molting (ecdysis). During ecdysis, insects experience a surge of hyperplastic growth in the epidermal layers underlying the cuticle (Rockstein, 1973). This mitotic surge requires extra energy to increase metabolism above normal resting standard metabolic rate.

The slope of whole animal $\dot{V}O_2$ over development, 0.82, differed slightly from the predicted interspecific mass-specific slopes of 0.67 - 0.75. This finding is in agreement with Kodric-Brown et al. (2006) who stated that intraspecific allometries of insects, i.e. stag beetles - *Neolucanus*, *Cyclommatus*, tend to have higher allometric exponents than their interspecific counterparts. The slope of mass-specific $\dot{V}O_2$ over development was -

0.18, which differed from the predicted interspecific mass-specific quarter power slope of -0.25. This also agrees with observations of intraspecific allometries having higher exponents (Kodric-Brown, 2006).

Newly hatched larvae of instar I have just completed embryogenesis and are utilizing energy stores. During embryogenesis, the majority of the imaginal tissues are created but proliferation continues, albeit minimally, during larval development (Heyland and Moroz, 2006). As expected, instar I larvae had the lowest body mass. They also the highest mass-specific $\dot{V}O_2$ and $\dot{V}CO_2$ rates, likely because they also had the highest ratio of metabolically active tissue mass to total body mass of any instars (see Chapter 4). There was no relationship between oxygen consumption and body mass in this instar.

In contrast to instar I, instar II showed a strong positive relationship between whole animal $\dot{V}O_2$ and body mass. The differences in $\dot{V}O_2$ of instar I to instar II larvae might be due to a decrease in the development of imaginal tissues.

Larval instar III to V stages followed the predicted metabolic allometric pattern in both whole animal and mass-specific $\dot{V}O_2$. The relationship of $\dot{V}O_2$ as a function of body mass maintained significance through these stages and the slopes (0.96, 1.00, and 1.02) of the whole animal $\dot{V}O_2$ approached isometry.

Prepupae had a higher whole animal $\dot{V}O_2$ than the adjacent stages, likely because of the vast increase in body mass gained during the feeding period immediately prior to this stage. Increases in feeding cause up to four-fold increases in metabolic rate in woollybear caterpillars (*Gynaephora groenlandica*) (Bennett, et al., 1999). During the prepupal stage of insects, the imaginal disks created in the embryo

begin to undergo mitosis and differentiation concurrent with the formation of the puparium, the hard outer case formed at the end of the prepupal stage (Heyland and Moroz, 2006). Mitotic increases have been associated with increases in $\dot{V}O_2$ (Rockstein, 1973).

The pupal phase had the lowest mass-specific $\dot{V}O_2$ rates of all the developmental stages. During metamorphosis, histolysis of the larval nervous tissue, epidermis, prothoracic glands, and parts of the digestive tract occur (Heyland and Moroz, 2006). Simultaneously, adult morphological structures are synthesized, e.g. tracheal muscles, eyes, wings, legs, genitalia and the adult nervous system (Heyland and Moroz, 2006). If the larger body mass of the prepupal and pupal stages were due to gender (i.e. the females making up the larger body masses), then there would probably not be a significant difference in mass-specific oxygen consumption, since the data does not indicate bimodality by visual inspection.

Metabolic Allometry, Body Surface Area and Body Mass

There were four methods of presentation of intraspecific allometric $\dot{V}O_2$ relationships in this study:

- whole animal $\dot{V}O_2$ as a function of body mass:
 - ($r^2=0.96$ ($p<0.0001$))
- mass-specific $\dot{V}O_2$ as a function of body mass:
 - ($r^2=0.54$, ($p<0.0001$))
- whole animal $\dot{V}O_2$ as a function of surface area:
 - ($r^2=0.85$, ($p<0.0001$))
- mass-specific $\dot{V}O_2$ as a function of surface area:

- ($r^2=0.47$, ($p<0.0001$))

Based on the regression analyses above, the method of plotting $\dot{V}O_2$ that yields the highest coefficient of determination is $\dot{V}O_2$ as a function of body mass. Whole animal $\dot{V}O_2$ is a more accurate method of describing intraspecific allometric relationships than mass-specific $\dot{V}O_2$ judging by the regression analyses above for both $\dot{V}O_2$ as a function of body mass and of surface area.

It would not be expected that metabolic rate would be related to total body surface area in insects as the cuticular surface area is not a factor with metabolic heat regulation as in homeotherms. However, metabolic rate may be related to the tracheal system surface area and this warrants further investigation as a possible method of data presentation for allometric metabolic allometry in insects.

Gender Effect on Oxygen Consumption

Whole animal $\dot{V}O_2$ was greater in females during the prepupal stage, when females are typically larger than males. Therefore, this is most likely not due to gender, but rather to differences in body mass. In adults, however, the whole animal $\dot{V}O_2$ rates were larger for the males than the females, despite being the smaller of the two sexes. By observation during the $\dot{V}O_2$ measurements, adult males showed elevated activity levels including trembling antennae and vigorous shaking of the body. Even with a 30-min calming period in the syringe before measurements, these animals did not settle into a quiescent state. This may be due to the air in the syringe being laden with the female pheromones. In contrast, the females remained quiescent at all times. The difference in adult $\dot{V}O_2$ may be attributed to the stimulation of pheromone receptors in males. Males have over 17,000 sensillas on their antennae that hold two different

odorant receptors stimulated by the female pheromones bombykol and bombykal (Syed et al., 2006). This receptor system is extraordinarily sensitive, with only one molecule of the female pheromone needed to stimulate the antennae receptors in the male (Syed et al., 2006). One male exhibited a $\dot{V}O_2$ of $15.12 \text{ ml O}_2 \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$ during a measurement period, which is 18 times higher than the average for females ($0.82 \text{ ml O}_2 \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$), and approached the metabolic rate of an active hummingbird ($15.58 \text{ ml CO}_2 \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$) (Powers and Conley, 1994). This raises an interesting question regarding male adult *Bombyx mori*'s maximum aerobic metabolic scope and energy store utilization, for example, is the tracheal system modified in males?

Carbon Dioxide Production and Respiratory Quotient

Mean mass-specific carbon dioxide production rates ranged from $0.24 \text{ ml CO}_2 \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$ in the pupal stage to $1.35 \text{ ml CO}_2 \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$ in instar I larvae. This is similar to VCO_2 in other insects such as adult desert beetles ranging from 0.08 to $1.71 \text{ ml CO}_2 \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$ (Lighton, 1991) and honeybees at an average of $0.41 \text{ ml CO}_2 \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$ (Lighton and Lovegrove, 1990). Many insects exhibit discontinuous ventilation patterns that vary during development and between species of insects (Levy and Schneiderman, 1966; Lighton, 1988, 2004, 2005; Hetz and Bradley, 2005; Gray and Bradley, 2006). The burst phase of discontinuous ventilation, when the spiracles are open, is variable - both intraspecifically and interspecifically. Inactive beetles were found to have four bursts within 20 minutes (Lighton, 1991) and four bursts within five minutes during activity. Lighton reported that at 25°C , adult ants had four bursts in 10 minutes. In the present study, there did not appear to be any spikes in $\dot{V}CO_2$ during the measurement periods. The individual measurements appeared consistent and therefore, the CO_2 bursts must

have occurred at a regular rate within the timed measurement periods or did not occur at all.

$\dot{V}O_2$ measurements were made on larvae throughout the instar and $\dot{V}CO_2$ measurements were made on larvae in the middle of the instar. Mean $\dot{V}O_2$ measured on larvae throughout each instar was assumed to be the equivalent of mean $\dot{V}CO_2$ measured from animals in the middle of each corresponding instar. Larvae undergoing ecdysis, i.e. from adherence to the surface via a silk pad to crawling out of the old cuticle, were not measured with either $\dot{V}O_2$ or $\dot{V}CO_2$. By observation, disruption of ecdysis increased mortality rates and thereby added confounding variables within the larval population. However, further investigations of $\dot{V}CO_2$ and $\dot{V}O_2$ during ecdysis may reveal interesting relationships.

Normal respiratory quotient (RQ) values in animals range from 0.7 (indicating fat catabolism) to 0.82 (indicating protein catabolism) and to 1.0 (indicating carbohydrate catabolism). However, RQ can fall well below 0.7 during the synthesis of carbohydrates via gluconeogenesis (Kleiber, 1975), or well above 1 during the synthesis of fat from carbohydrates (Popjak, 1957; Kleiber, 1975). RQ's below 0.70 occur during metamorphosis in insects (Kleiber, 1975) and some have compared this state in insects to hibernating mammals (Walsburg and Hoffman, 2005).

Based on RQ values, larval instar I of *Bombyx mori* primarily metabolized carbohydrates from embryogenesis. A switch to fat metabolism occurred during the next three larval stages. This might be attributed to food deprivation the twelve hours prior to oxygen consumption measurements. Similarly, carbohydrates are utilized in early

development of crustacean embryos and a switch to fat and protein breakdown occurs in subsequent stages (Needham, 1933).

Prepupal RQ increased above 1.0 during preparation for metamorphosis. An RQ above 1.0 can be an indicator for lipogenesis (Kleiber, 1975). During the prepupal stage, the insect has undergone an intensive feeding stage and synthesizes fat stores for the remainder of its life cycle. The RQ of 0.86 during metamorphosis in *Bombyx mori* in the present study could be explained by protein metabolism or a mixture of carbohydrate and lipid metabolism. Metamorphosing pupae undergo histolysis and histogenesis simultaneously. The RQ during this stage could possibly be an indicator of the catabolism of all three nutrient sources. RQ values for two species of adult insects were found to be 0.83 in ants and 0.86 in honeybees (Lighton, 1998; Lighton and Lovegrove, 1990).

Heart Rate

No relationship exists between body mass and heart rate in instar IV and V. This is perhaps not surprising, since the circulatory system in insects is not used for gas exchange. In insects, the exchange of gases is accomplished by diffusion or convection through the tracheal system. The circulatory system transports non-respiratory materials such as nutrients, waste products, hormones as well as other molecules and therefore blood flow might not be expected to be as closely related to oxygen consumption as in animals dependent upon circulation for gas exchange. However, further investigations on the remainder of the developmental stages would increase the magnitude of the range of body mass and may reveal allometric relationships throughout development.

The heart rate during activity or during specific dynamic action may also reveal relationships to body mass.

These observations collectively support the idea that intraspecific allometric relationships of metabolism generally follow the trend of the interspecific allometric relationships of adult metazoans, but also have significantly different relationships within individual instars. Further investigations should be made to determine when the developing larvae switch from hyperplastic growth to hypertrophic growth to ascertain whether these processes have an influence over the changes in the rates of $\dot{V}O_2$ during development. Therefore, I support my initial hypothesis of the differences in intraspecific developmental metabolic allometric patterns by lifecycle stage over the course of development in *Bombyx mori*.

Conclusion

Body mass, whole animal $\dot{V}O_2$ and mass-specific $\dot{V}O_2$ changes over development were found to be in agreement with previous literature on oxygen consumption of developing insects and with the interspecific “mouse to elephant curve”.

However, regression analysis of oxygen consumption as a function of body mass of individual developmental stages revealed relationships that were significantly different from the overall regression throughout development. The significant differences in the slopes of the individual instars to the overall slope during development raises an interesting point. For example, if the overall developmental slope is “A”, but is comprised of components “ b_1 , b_2 , b_3 , etc.”, that have statistically significantly different slopes from the overall “A”, this generates a question of the validity of the use of the overall slope “A” for interspecific comparisons. At a minimum, I argue that this overall

slope should not be used for the comparison of growing animals that are undergoing complex developmental stages and should be presented as individual developmental stages of whole animal $\dot{V}O_2$ as a function of body mass - which is the most appropriate method of presenting intraspecific metabolic allometric relationships.

The overall developmental slope was 0.82 and the individual slopes contributing to this average slope value were, i.e. 0.99, 0.96, 1.00, and 1.02. There were two developmental stages that did not have significant slopes, i.e. larval instar I and pupal, but were included in the overall regression analysis throughout development and thereby lowered the overall slope. If the regression analysis would have been performed on larval instar II through the prepupal stage, the overall slope would have approached isometry. Consequently, the overall developmental slope value of 0.82 is a statistical artifact in this study.

Within a developmental stage, especially the pupal stage, physiological states vary. These changes may cause fluctuating relationships of metabolic rate as a function of body mass *within* developmental stages that might contribute statistically significant allometric slope coefficients. Therefore, further investigations within each developmental instar are warranted to determine if the individual developmental stage allometric slopes are also statistical artifacts.

CHAPTER 3

METAMORPHOSIS, OXYGEN CONSUMPTION AND WATER LOSS IN THE SILKWORM, *Bombyx mori*: INVESTIGATION OF THE ROLE OF THE SILK COCOON

Introduction

Holometabolous insects like the silkworm, *Bombyx mori*, have a brief life cycle (~55-60 days) consisting of five instars separated by larval-to-larval molts (ecdysis). At the end of the fifth larval instar, the insects enter a feeding stage that stores energy for the brief remainder of the life cycle. At the end of the feeding period, the absence of juvenile hormone allows the neurosecretory hormone, ecdysone, to initiate metamorphosis, marking the beginning of the prepupal stage. The prepupa locates a suitable place to spin a cocoon around its body. The imaginal tissues of the prepupa, dormant since their creation during embryogenesis, begin proliferation (Ohtaki, 1986; Nijhout and Grunert, 2002). For approximately five days, the prepupa spins a silk cocoon around its contracted body, the larval-prepupal molt occurs and tanning of the outer cuticle follows. Histolysis and histogenesis occur simultaneously during metamorphosis. The silk glands are the first to undergo histolysis, possibly from programmed cell death followed by phagocytosis (Rockstein, 1973; Ohtaki, 1986). The proliferation of imaginal tissues of the wings, legs, antennae, thorax and abdomen are followed by the histolysis of the midgut and the mandible (Ohtaki, 1986).

The silkworm secretes silk strands from labial glands that are analogous to salivary glands in other larval insects (Asakura et al., 2006). The high tensile strength characteristics of silk make it desirable for use in biomedical tissue engineering (Hakimi et al., 2006). Silk is made up of two major components - microfilaments of an insoluble

protein (fibroin), covered with a soluble adhesive protein (sericin) that provides structural support to the cocoon (Zhou et al, 2000; Hakimi et al., 2006). In addition to the fibroin and sericin proteins, there are other minor components such as small proteins, lipids, and carbohydrates (Gauthier, et al., 2004). The function of the cocoon in insects such as the leek moth (*Acrolepiopsis assectella*) and the honey bee (*Apis linnaeus*) during metamorphosis is generally thought to provide protection from predators, biodegradation, and dehydration (Gauthier et al, 2004; Sutherland et al, 2006). However, no study has measured the influence of the cocoon on oxygen consumption or water vapor loss.

In this study I investigated the oxygen consumption of prepupal, pupal and adult stages of the silkworm, *Bombyx mori* to test the hypotheses that the cocoon reduces the diffusion rate of oxygen and that the cocoon limits the rate of water vapor diffusion during metamorphosis. Measurements were made of $\dot{V}O_2$ in pupae with and without their cocoons as well as a sham group with an opened cocoon. I also measured the rate of body mass loss over time in low relative humidity for intact and naked pupae to determine if the cocoon influenced the rate of water loss.

Materials and Methods

Animal Husbandry

Fertilized eggs of the silkworm, *Bombyx mori*, were obtained from Carolina Biological Supply, Mulberry Farms and Coastal Silkworms. Upon arrival in the laboratory, the eggs were placed in Petri dishes and incubated in a transparent incubator at a temperature of 25 ± 0.5 °C with a relative humidity of $60 \pm 5\%$. The incubators were illuminated with a LD 12:12 photoperiod. The Petri dishes were

examined for hatched larvae each morning, since hatching occurs in the morning due to circadian rhythms of metabolism and eclosion hormone secretion (Fugo et. al, 1984). Newly hatched instar I larvae were transferred into date-labeled Petri dishes. All larval instars were fed an artificial silkworm diet obtained from Carolina Biological Supply in the afternoon and food remaining in the Petri dishes was removed daily in the evening.

Respirometry System

Many investigations of metabolic allometry have measured oxygen consumption ($\dot{V}O_2$), an accepted indirect method of estimating aerobic metabolic rate (McMahon, 1973; Else and Hubert, 1981; German, 1989; West, et. al, 1997; West, 2002; Kozlowski, 2003; Brown, 2004; Economo, 2005, and others). Closed system respirometry was utilized in this study to measure both $\dot{V}O_2$ and carbon dioxide production ($\dot{V}CO_2$) during the entire life-cycle of *Bombyx mori*. The larvae and pupae were placed in glass syringes, varying in Volume depending on larval size (Table 1). A 20-gauge needle was fastened securely onto the syringe and inserted into a rubber stopper, thus creating a watertight, gas-tight closed respirometer of known Volume (Figure 2.1). All measurements were made at 25 ± 0.5 °C.

Measurements of $\dot{V}O_2$ and $\dot{V}CO_2$ were performed daily on larvae and pupae pre-weighed to the nearest milligram. All of the holding chambers, i.e. syringes, water bath, incubator, were transparent and measurements were made during daylight hours to prevent disruption of normal circadian rhythms. Hormonal activity has been linked to the light-dark cycle in silkworms (Fugo 1984), and changes in light and / or dark has also been shown to alter the $\dot{V}O_2$, heart rate and / or cause heart rate reversals in other

insect species (Stusek, 2000). All measurements were made approximately 12 hours after feeding to avoid specific dynamic action increases in $\dot{V}O_2$ (Bennett, et al., 1999).

The larvae were retrieved from the incubator and placed in the gas-tight glass syringe for 30 min before a baseline $\dot{V}O_2$ or $\dot{V}CO_2$ measurement was made to ensure that larvae were in a quiescent state and maintaining a standard metabolic rate before measurements were taken. Larvae were periodically viewed during the holding and measurement period to confirm their inactivity. Any active larvae were removed from the experiment. Individual larvae were measured three times each day and mean $\dot{V}O_2$ or $\dot{V}CO_2$ was calculated for each larvae. The syringe was refreshed with air at $25 \pm 0.5^\circ\text{C}$ between each reading. A measured Volume of air (~ 0.5 ml) was injected directly from the syringe containing the animal into the oxygen or carbon dioxide analyzer at specified time periods, as indicated for various life stages (see Chapter 2).

Oxygen Consumption Measurements and Calculations

Oxygen levels in 0.5 ml gas samples injected directly from the syringe respirometers were measured with a thermostatted Clark-type oxygen electrode (Radiometer, Model PHM 72 Mk-2). Each day, barometric pressure was recorded and pO_2 of ambient air was calculated by subtracting the water vapor pressure. The electrode was calibrated with a two-point calibration using atmospheric air and pure nitrogen prior to each experiment and after each measurement period throughout the day. Oxygen consumption was calculated using the formulas described in Chapter 2. $\dot{V}O_2$ rates are reported as whole animal ($\text{ml } O_2 \cdot \text{hr}^{-1}$) and mass-specific ($\text{ml } O_2 \cdot \text{g}^{-1}\text{hr}^{-1}$). Mean ± 1 standard error (s.e.) was calculated for body mass and mass-specific $\dot{V}O_2$ as a function of developmental stage.

$\dot{V}O_2$ in Pupae: Cocoon Manipulations

Three experimental groups of pupae were selected for $\dot{V}O_2$ measurements to determine if the cocoon was a limiting factor for oxygen consumption. All $\dot{V}O_2$ measurements were performed as described above. The control group consisted of intact, undisturbed pupae in their cocoon. “Body mass” in this group comprised pupae and its surrounding cocoon, pre-weighed to the nearest mg. In the sham group each pupa was pre-weighed to the nearest mg and carefully removed from its cocoon through a thin opening made with a sharp razor blade. The naked pupa was weighed to the nearest mg and then placed back into its cocoon, which was then closed by black-braided non-absorbable silk sutures (size 8-0). For consistency, body mass in this group comprised pupae and its surrounding cocoon. In the final group each pupa was pre-weighed to the nearest mg, removed from its cocoon, the naked pupa was measured to the nearest mg, and then subjected to metabolic measurements without its cocoon. Body mass in this group comprised pupae and its surrounding cocoon.

Cocoon Water Vapor Diffusion Properties

Undisturbed pupae (control group) and naked pupae (cocoon removed) were placed into a (1L) water jacketed glass chamber held at a constant relative dry humidity of 30-35% and temperature of $24 \pm 1.0^\circ\text{C}$. Air from a gas line was circulated through the top of the chamber and the outflow air was collected in a sealed plastic bag and measured with a hygrometer. The control group consisted of intact pupae in their cocoons that were pre-weighed to the nearest mg. In the experimental group, pupae were carefully removed from their cocoon by a thin opening made by a sharp razor blade. As with the controls, each naked pupa of the experimental group was weighed to

the nearest mg but remained without its cocoon for the measurement period. The control group, naked pupae, and the empty cocoons from the naked pupae were placed on a Petri dish inside the water jacketed chamber. The controls, naked pupae, and the cocoons removed from the naked pupae were weighed to the nearest mg at T= 0.5, 2, 24, and 48 h to determine percent change from baseline and rate of total mass change. The bottom layer of the glass water jacketed chamber was filled with Drierite to minimize the humidity in the container and the lid of the glass chamber was sealed with parafilm. During the measurements, when the glass container was opened, the Drierite was stirred to ensure uniformity in water vapor absorbance. The top layer of the Drierite indicated water absorbance by changing slightly from blue to purple after 24 h and was replaced with a new layer of Drierite.

Analyses and Statistics

Mean $\dot{V}O_2 \pm 1$ standard error (s.e.) was calculated for body mass across the life cycle. A non-linear regression analysis was performed on $\dot{V}O_2$ as a function of body mass data in the prepupal, pupal and adult silkworm moths. Mean $\dot{V}O_2 \pm 1$ standard error (s.e.) was calculated for the control, sham and naked pupae, and was expressed as both whole animal and mass-specific $\dot{V}O_2$. A one-way ANOVA was performed on these groups to determine significant differences in mean $\dot{V}O_2$ between groups.

Percentage changes in body mass from the baseline were calculated for the control and experimental groups as well as the empty cocoons. Data were arcsine transformed prior to statistical testing. A two-way ANOVA was performed to determine if mean was influenced by mass loss by group, time or an interaction of group and time.

An independent t-test was performed between the groups (i.e. control and experimental) at each time period. An alpha level of 0.05 was adopted for all statistical decisions.

Results

Body Mass

Body mass increased during development from larval instar I (mean and s.e. = $.00050 \text{ g} \pm .00002 \text{ g}$) to the prepupal stage ($1.83 \text{ g} \pm 0.10 \text{ g}$) and then decreased with the pupal ($0.79 \text{ g} \pm 0.02 \text{ g}$) and adult ($0.38 \text{ g} \pm 0.03 \text{ g}$) stages (Figure 3.1). The rate of body mass change ranged from an increase of $0.00005 \text{ g} \cdot \text{day}^{-1}$ in larval instar I to a $0.23 \text{ g} \cdot \text{day}^{-1}$ increase in prepupal stage (Figure 3.2A). The greatest decrease of body mass was $-.09 \text{ g} \cdot \text{day}^{-1}$ in the adult stage. The rate of mean body mass change as a function of developmental stage was greatest with larval instar IV at 23%, and the greatest decrease in body mass was in the adults at -26% (Figure 3.2B).

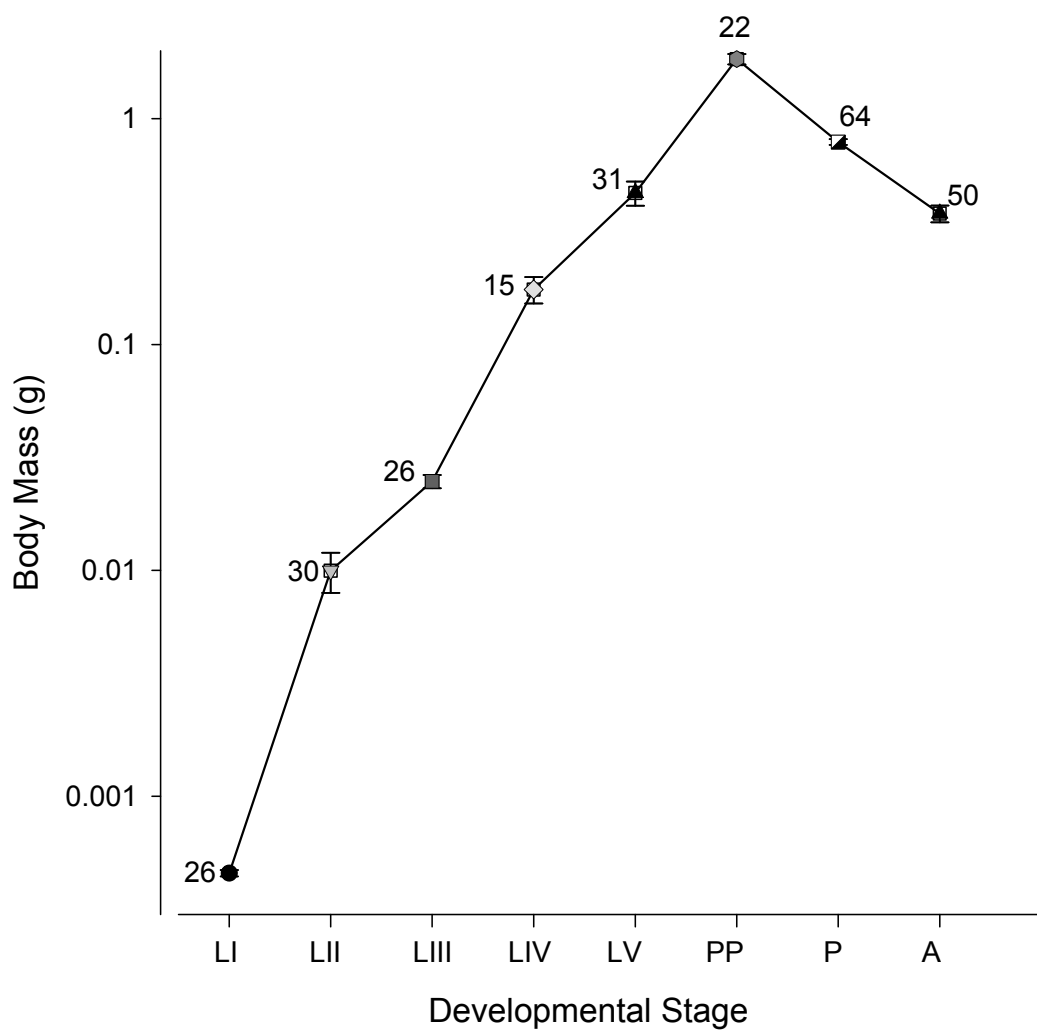


Figure 3.1: Body mass throughout the life cycle of *Bombyx mori*. Mean \pm 1 s.e., n values, and SNK groups are differentiated by different symbols.

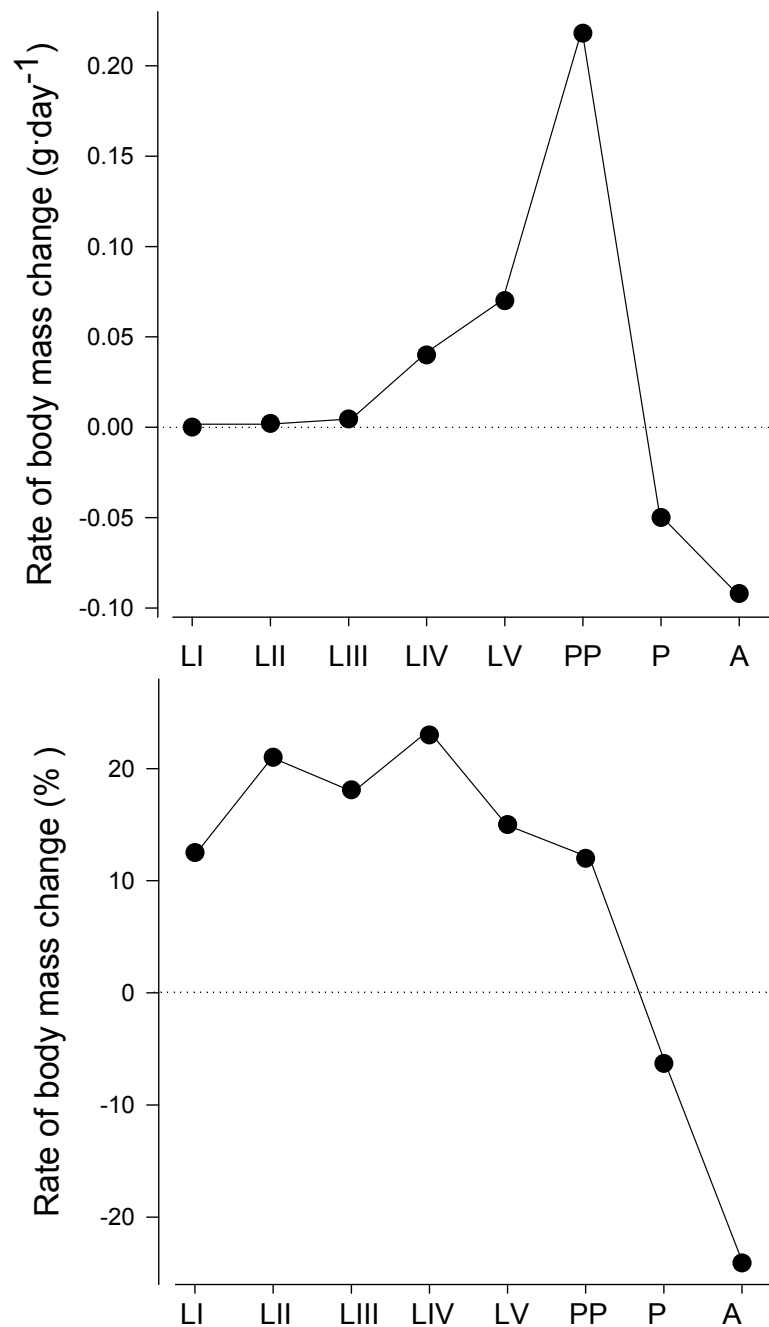


Figure 3.2: Rate of body mass change, expressed as $\text{g}\cdot\text{day}^{-1}$ (A) and % of mean body mass (B) as a function of developmental stage in *Bombyx mori*.

During metamorphosis, the prepupal larva undergoes a larval-pupal molt and sheds the prepupal skin inside of the cocoon. The mean non-living mass of the pupae (i.e. cocoon + dehydrated prepupal skin) encompasses 16.20 % + 1.02 % of the mean body mass of undisturbed pupae. The dehydrated prepupal skin inside of the cocoon during metamorphosis encompasses a negligible amount of the total body mass ($3\% \pm 0.10\%$) and was grouped with the cocoon mass for the purposes of this study.

$\dot{V}O_2$ Spanning Metamorphosis

Prepupal larvae had a mean $\dot{V}O_2$ of 0.77 ± 0.08 ml $O_2 \cdot h^{-1}$ with a coefficient of determination (r^2) of 0.66 and a slope (b) of 1.49 ($p < 0.01$) (Figure 3.3). Pupae had a mean whole animal $\dot{V}O_2$ of 0.21 ± 0.01 ml $O_2 \cdot h^{-1}$ with an r^2 value of 0.04 and a slope not significantly different from zero. The data for adult males was not used in the regression analysis due to the sustained and variable activity during the $\dot{V}O_2$ measurements resulting from potential stimulation by female sex pheromones. Adult females had a mean whole animal $\dot{V}O_2$ of 0.62 ± 0.08 ml $O_2 \cdot h^{-1}$ with an r^2 value of 0.25 and a slope not significantly different than zero.

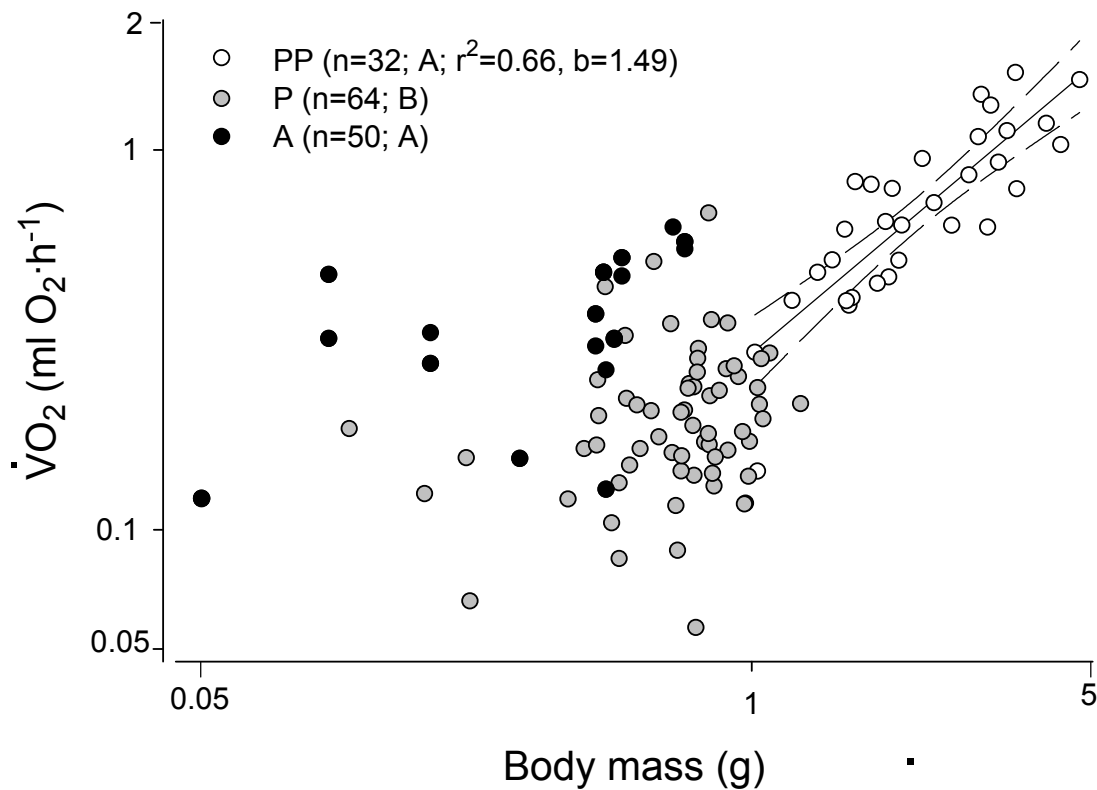


Figure 3.3: $\dot{V}O_2$ as a function of body mass of prepupal, pupal and adult *Bombyx mori*. Statistically significant linear simple regression lines with 95% confidence intervals, n values, statistically significant SNK groups (designated by letters A, B), and regression coefficients are provided.

A one-way ANOVA showed a significant difference in whole animal mean $\dot{V}O_2$ among the groups ($p < 0.0001$) and an SNK separated the means into two statistically significant groups: A: prepupae, adult; B: pupae.

$\dot{V}O_2$ in Pupae: Cocoon Manipulations

Mean whole animal and mass-specific $\dot{V}O_2$ for the control, sham and naked groups were not statistically different ($p = 0.26$), and ($p = 0.13$) respectively (see Table 3.1; Figure 3.4).

	Control	Sham	Naked Pupa
Mean whole animal $\dot{V}O_2 \pm \text{s.e. in ml O}_2 \cdot \text{h}^{-1} (n)$	0.19 ± 0.01 (43)	0.16 ± 0.01 (22)	0.18 ± 0.01 (24)
Mean mass-specific $\dot{V}O_2 \pm \text{s.e. in ml O}_2 \cdot \text{g}^{-1} \text{h}^{-1} (n)$	0.22 ± 0.01 (43)	0.21 ± 0.02 (22)	0.24 ± 0.02 (24)

Table 3.1: Mean $\dot{V}O_2 \pm \text{s.e.}$ of control (intact cocoon), sham (removed from cocoon and reinserted into cocoon) and naked pupae (removed from cocoon) of metamorphosing *Bombyx mori* pupae at $25 \pm 0.5^\circ\text{C}$.

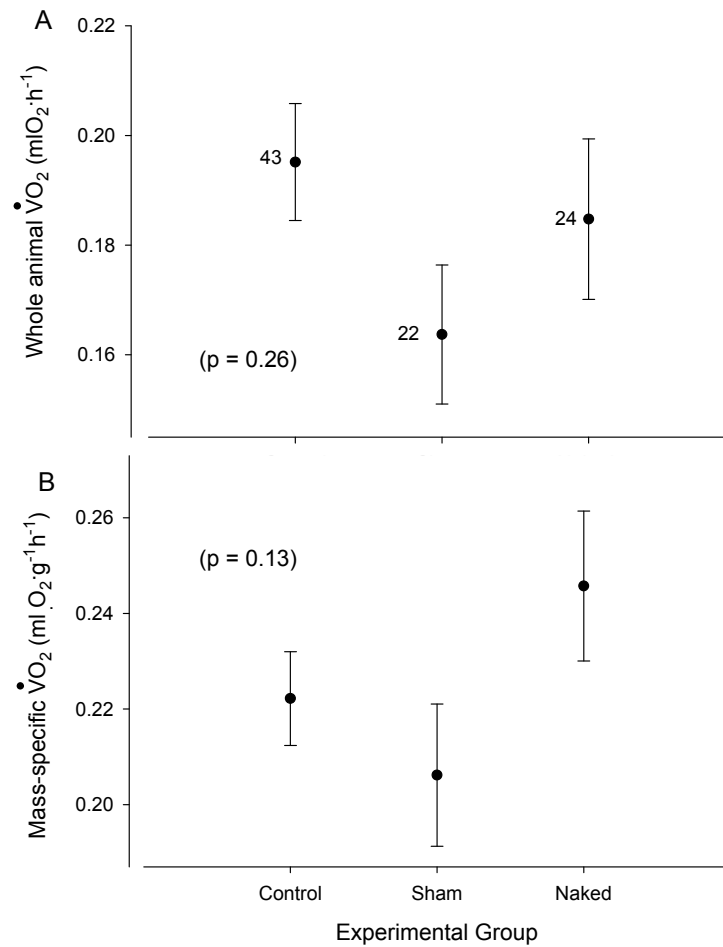


Figure 3.4: (A) Whole animal $\dot{V}O_2$ and (B) mass-specific $\dot{V}O_2$ of control (pupa plus cocoon), sham (opened cocoons) and naked groups of metamorphosing *Bombyx mori*. Mean ± 1 s.e., p values and n values are shown.

Mean mass-specific $\dot{V}O_2$ for the intact pupa group (including cocoon mass) and the same intact pupa group with the cocoon mass excluded was $0.24 \pm 0.02 \text{ ml O}_2 \cdot \text{h}^{-1}$ and $0.28 \pm 0.02 \text{ ml O}_2 \cdot \text{h}^{-1}$, respectively (Figure 3.5). These two groups were not significantly different ($p = 0.18$).

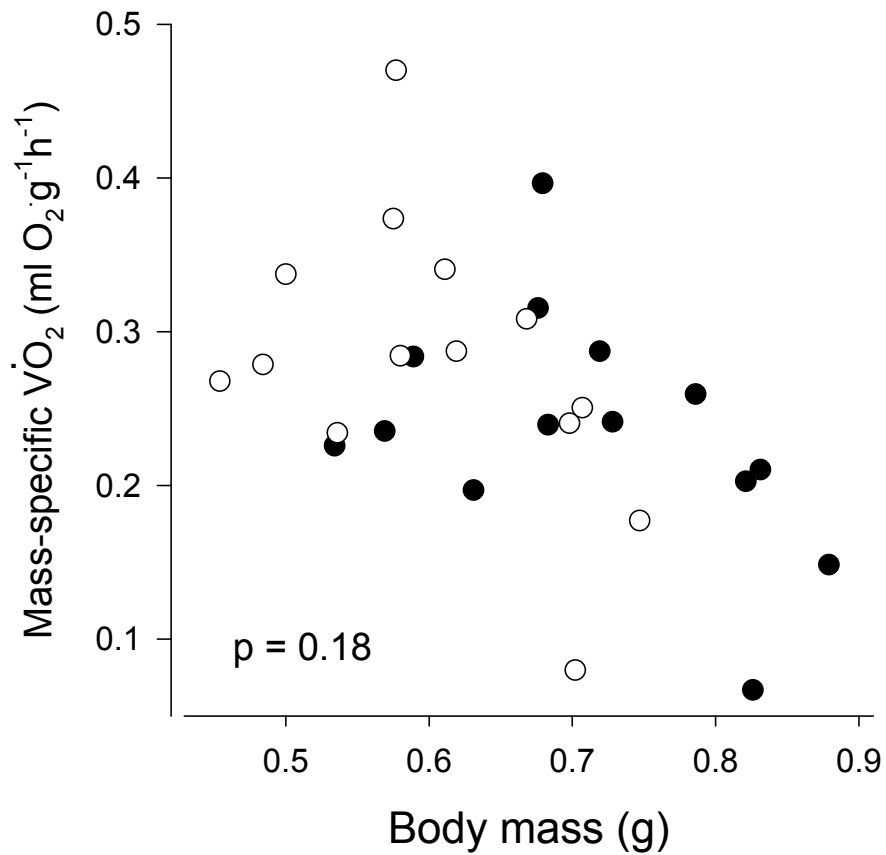


Figure 3.5: Mass-specific $\dot{V}O_2$ as a function of body mass in developing *Bombyx mori* in intact pupae (with cocoon mass) and with naked pupae (minus cocoon mass). N and p values are shown.

Time (h)	0	0.5	2	24	48
	Body mass (mean g \pm s.e.)				
Control n=8	0.89 \pm 0.04	0.88 \pm 0.04	0.88 \pm 0.04	0.87 \pm 0.04	0.86 \pm 0.04
Naked pupa n=7	0.66 \pm 0.04	0.66 \pm 0.04	0.66 \pm 0.04	0.65 \pm 0.03	0.64 \pm 0.03
Cocoon n=7	0.15 \pm 0.01	0.15 \pm 0.01	0.15 \pm 0.01	0.15 \pm 0.01	0.14 \pm 0.01

Table 3.2: Body mass loss of intact and naked pupae (removed from cocoon) and empty cocoons of metamorphosing *Bombyx mori* pupae over time at a low relative humidity (30-35%) and at 24 \pm 1.0 °C.

Cocoon Influence on Water Loss

At the beginning of a period of desiccation (0 h), the control group had a mean mass of 0.89 g \pm 0.04 g. After 48h, body mass had declined to 0.86 g \pm 0.04 g, or about a 3.1% overall body mass loss (Table 3.2).

The mean body mass of the naked pupa at 0 h was 0.66 g \pm 0.04 g and after 48 h, ended at a mass of 0.64 g \pm 0.03 g, amounting to a 3.3% overall body mass loss. The mean mass of the empty cocoon group at 0 h was 0.15 g \pm 0.01 g and after 48 h, ended at a mass of 0.14 g \pm 0.01 g, a 2.4% overall body mass loss. A two-way ANOVA

showed no significant difference among the mean masses of the experimental groups ($p=0.60$), but did show a significant difference in the mean values between different times ($p<0.001$) (Figure 3.6). However, there was no statistically significant interaction between group and time ($p=0.37$). Multiple comparison procedures (SNK) separated the following mean mass loss relative to time as: A: 0.00 h - 2.00 h; B: 2.00 h -24.00 h; C: 24.00 h -48.00 h.

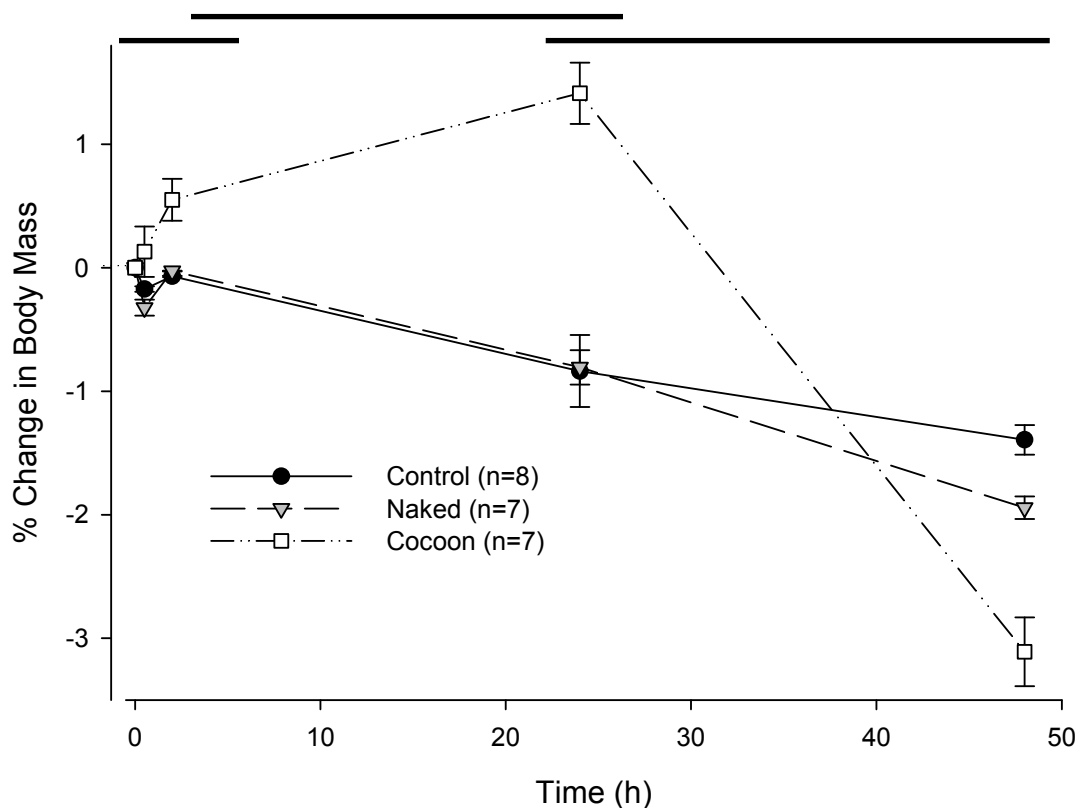


Figure 3.6: Percent body mass change over time of three experimental groups (control, naked and empty cocoons) of metamorphosing *Bombyx mori* held in constant dry relative humidity of 30-35% at 23-25°C. Mean \pm 1 s.e., SNK groups are indicated by horizontal lines (two-way ANOVA, $p<0.001$), and n values are shown).

The rate of body mass loss of control pupae, naked pupae and empty cocoons over time showed an initial spike after 30 minutes of a mean and s.e. in the naked group

of $3.6 \pm 0.4 \text{ mg}\cdot\text{h}^{-1}$ and in the control group of $3.5 \pm 0.3 \text{ mg}\cdot\text{h}^{-1}$ and then decreased to $0.15 \pm 0.12 \text{ mg}\cdot\text{h}^{-1}$ at 2 h in the naked group and $0.65 \pm 0.30 \text{ mg}\cdot\text{h}^{-1}$ in the control group and thereafter stabilized after 2 h for the remainder of the measurement period (Figure 3.7).

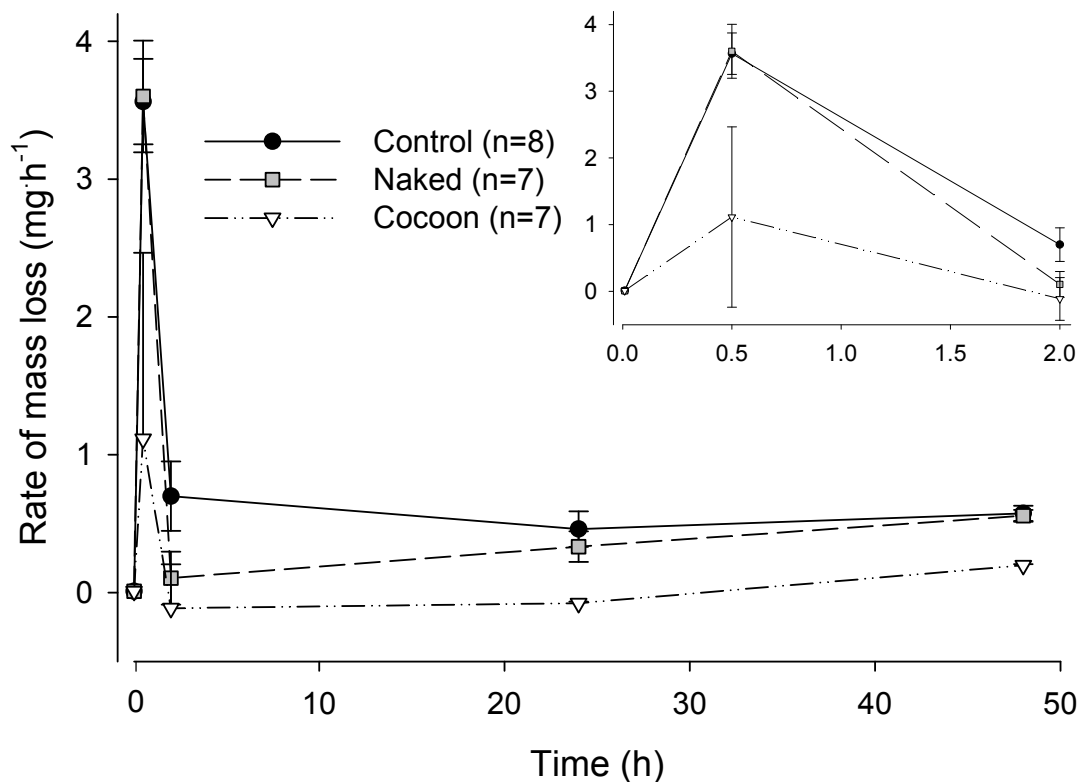


Figure 3.7: Rate of body mass loss of control pupae, naked pupae and empty cocoons of *Bombyx mori* over time at low relative humidity (30-35%) and at $24 \pm 1^\circ\text{C}$. Mean \pm 1 s.e. and n values are shown.

Discussion

Body Mass Changes

A progressive body mass increase occurred in *Bombyx mori* through its larval instars. The greatest rate of absolute body mass increase occurred between larval instar V and the prepupal stage. This corresponds with the eating phase that stores

energy for the remainder of the lifecycle. Body mass started to decline in the pupal stage and was reduced from the prepupal high by about 80% in the final adult state. Therefore, once these insects reach a maximum weight in the prepupal stage, metamorphosis commences and simultaneous processes occur such as production of adult tissues and apoptosis of larval tissues. The pupae is a closed system where energy is conserved and energy requiring processes (i.e. maintenance of tissues) as well as evaporative water loss cause the body mass to decrease with age.

$\dot{V}O_2$ Spanning Metamorphosis

The prepupal stage had a higher whole animal $\dot{V}O_2$ than the pupal stage, likely because of the vast increase in body mass gained during the feeding period immediately prior to this stage. Increased feeding can increase the metabolic rate up to four-fold in woollybear caterpillars (*Gynaephora groenlandica*) (Bennett, et al., 1999). During the prepupal stage of insects, the imaginal disks created in the embryo begin to undergo mitosis and differentiation concurrent with the formation of the puparium, i.e. the hard outer case formed at the end of the prepupal stage (Heyland and Moroz, 2006).

The pupal phase had the lowest whole animal $\dot{V}O_2$ rates of all the developmental stages. Histolysis (via apoptosis), phagocytosis, and histogenesis occur simultaneously in the pupal stage, and distinguish it from larval and adult stages. Histolysis of the larval nervous tissue, epidermis, prothoracic glands, and parts of the digestive tract occurs and simultaneously, adult morphological structures are synthesized, e.g. tracheal muscles, eyes, wings, legs, genitalia and the adult nervous system (Heyland and Moroz, 2006). These processes, along with decreasing body mass from prepupa to

adult, may contribute to a different composition of metabolically active tissues within these three stages that contribute to the observed differences in metabolic rates.

The increased $\dot{V}O_2$ is due primarily to the behavior of the adult moths. At the end of the sedentary physiological state of the pupal stage, the adult moths emerge with a sole function of reproduction. The female moths undergo minimal activity whereas the male moths are highly active due to numerous sensilla located on their antennae that are stimulated by the female pheromones bombykal and bombykol (Sakuri, et al., 2004).

Influence of Cocoon on $\dot{V}O_2$

Control, sham and naked pupa showed no significant differences in either whole animal or mass-specific $\dot{V}O_2$ within the four hour measurement period. The cocoon thus does not limit diffusion of oxygen into the cocoon nor does it limit oxygen consumption – at least in the period when measurements were made. Most insects undergo discontinuous respiration cycles that are characterized by the movements of the spiracles. There is no exchange of gases during the closed phase, minimal exchange during the flutter phase and maximum exchange during the open phase (Tenney, 1985; Gibbs and Johnson, 2004). The spiracles of the naked pupae might compensate when the naked pupa is removed from the cocoon by entering the closed phase of the discontinuous respiratory cycle. However, if this did occur during these experiments, it was not at an important cost of energy as the $\dot{V}O_2$ was not significantly different from controls. Further investigations on long term effects of cocoon removal as well as measurements of spiracular movements of the naked pupae are warranted to determine if in naked pupae the tracheal system compensates for the lack of a cocoon by closing

the spiracles, a process that would be stimulated by an increase in the pO_2 gradient to maintain the $\dot{V}O_2$ at a homeostatic set point.

In Chapter 2, I measured $\dot{V}O_2$ of undisturbed pupae, determining the appropriate method for measuring $\dot{V}O_2$ during metamorphosis. Since there was no significant difference between the $\dot{V}O_2$ of the undisturbed pupae and the naked pupae, measuring $\dot{V}O_2$ of undisturbed pupa inside of a cocoon is valid.

When measuring $\dot{V}O_2$ on metamorphosing pupae, there is not a significant difference in calculating $\dot{V}O_2$ (mass-specific) utilizing body mass including non-living mass (cocoon and prepupal cuticle mass) or by subtracting the non-living mass (cocoon and prepupal cuticle mass) that accounts for a mean and s.e. of 16.20 % + 1.02 % of the total body mass. Therefore, reporting $\dot{V}O_2$ values of metamorphosing pupae utilizing the entire body mass (living and non-living) is valid.

Role of Cocoon in Desiccation Resistance

If the cocoon of pupal *Bombyx mori* helps to conserve water, then the naked pupae should undergo a more rapid mass loss than the intact pupae. Yet, both the intact control pupae and the naked pupae showed the same pattern and rate of water loss over 48 h. These data do not support the idea that the insect cocoon, (i.e. *Acrolepiopsis assectella*) protects pupa from desiccation (Gauthier, 2004). Although pupae lost water during this experiment, rates of water loss were not different in naked and intact pupae. There were significant differences between the group's water losses over time. Each measurement period after the 30-min measurement was significantly different from the other measurement periods and therefore, the diffusion of water over time is consistent between the intact control and the naked pupae.

Conclusion

The prepupal, pupal and adult stages are stages of development of *Bombyx mori* possessing variations in biochemistry, physiology and behavior and these variations may attribute to differences in metabolic rate. I questioned the validity of measuring the $\dot{V}O_2$ of pupa in intact cocoons as opposed to naked pupa. Since there were no significant differences between $\dot{V}O_2$ or body mass loss between intact pupa and naked pupa, and there were no significant differences in either whole animal or mass-specific $\dot{V}O_2$ of intact pupae and the same intact pupae with the cocoon (and prepupal cuticle) mass subtracted from the body mass - measuring the $\dot{V}O_2$ of intact pupa should be considered a legitimate method. When measuring the $\dot{V}O_2$ in intact cocoons, the cocoon did not influence either $\dot{V}O_2$ or the rate of mass loss. The *Bombyx mori* cocoon may provide protection from dessication in more extreme conditions than were investigated, i.e. wind, or may only function to provide mechanical protection during metamorphosis.

CHAPTER 4

BODY COMPOSITION VARIATION DURING DEVELOPMENT OF *BOMBYX MORI*

Introduction

The amount of metabolically active tissues per body mass plays a role in the fluctuations of the relationship of $\dot{V}O_2$ as a function of body mass (Schmidt-Nielsen, 1984; Wang et al., 1992; Hulbert and Else, 2005). All animals can be viewed as having multiple body compartments with varying degrees of energy consumption, as reflected in oxygen uptake. The organs having the highest metabolic rate may not increase in the same proportion with increasing size as the organs that contribute least to the metabolic rate, such as bone (Hulbert and Else, 2005; Hunt Von Herbing, 2006). An organism can be subdivided into five major classes: atomic, molecular, cellular, tissue-system and the whole body - with each characterized by allometric relationships between metabolic rate and body mass (Wang et al., 1992). Animals can more simply be compartmentalized by lean body mass (metabolically active) and fat body mass (metabolically inert) (Wang et al., 1992; Hunt Von Herbing, 2006). Lean body mass has a higher correlation to metabolic rate than whole organism mass (Hunt Von Herbing, 2006). The proportion of lean body mass determines the metabolic rate, with larger animals having a lower mass specific $\dot{V}O_2$ due to an increasing percentage of metabolically inert tissue of total body mass. The individual mass-specific $\dot{V}O_2$ of organs from heavier body mass animals is lower than that of smaller body mass animals (Wang et al., 1992; Hunt Von Herbing, 2006). The cause might be due to an organ mass increase in metabolically inert tissue, i.e. adipose, as opposed to gains in metabolically active tissue mass, i.e. muscle.

One of the highest metabolically active tissues is muscle, especially the flight muscles of insects (Downer and Matthews, 1976; Baldwin and Hakim, 1991; Gibellato and Chamberlin, 1994). The insect midgut is another highly metabolic organ with a mitochondrial and rough endoplasmic reticulum-rich columnar epithelial layer and outer circular and longitudinal smooth muscle layers (Mandel et al., 1975, 1980; Bradley, 1984; Parenti et al., 1985; Baldwin and Hakim, 1991; Gibellato and Chamberlin, 1994; Jouni and Wells, 1996; Grant, 2006). Goblet cells are dispersed throughout the columnar epithelium and are rich in mitochondria and play an active role in ion transport (Baldwin and Hakim, 1991; Gibellato and Chamberlin, 1994). Heart and nervous tissues also have high metabolic tissues (Hunt Von Herbing, 2006). Cuticle and total body lipid content, on the other hand, should be considered low metabolically contributing tissues by comparison (Schmidt-Nielsen, 1984; Hunt Von Herbing, 2006). However, in insects, the cuticle can be considered a high metabolic tissue during ecdysis as there is a mitotic surge when the new cuticle is formed (Rockstein, 1973; Ganga, 2003). However, in the present study, $\dot{V}O_2$ was not measured during ecdysis and therefore, the cuticle will be considered a low metabolic tissue as compared to other tissues.

Water does not directly contribute to aerobic respiratory rates. As with water, the lipids of the body should be considered to be in the metabolically inert group of body compartments. In insects, lipids primarily serve as cellular membrane structure and for the precursor materials of the steroidal hormone, ecdysone (Fernando-Narnakulasurya et al., 1988). The most common forms of lipids are phospholipids, sphingolipids, steroids, and glycerides which make up approximately 90% of lipids within an insect body (Fast, 1964; Allen, 1976; Patel et al., 2004; CanaVOso et al., 2004). Most lipids in

the insect body are stored in a dispersed organ termed the fat body. The fat body is analogous to both the liver and adipose tissue in vertebrates and functions in energy storage and production of hormones and proteins (CanaVOso et al., 2004; Patel et al., 2004). A major change in the fat body during the prepupal stage of metamorphosis is the switch from active lipid storage to the utilization of lipid reserves most likely caused by the ecdysone peak (Prasad et al., 1986). The fat body of insects is considered to be a high metabolic organ, but the total lipid content of the body including the lipid content of the fat body is metabolically inert.

The metabolic potential of mitochondria is directly related to the amount of respiratory enzymes (Watanabe and Williams, 1950). Cytochrome c oxidase is the enzyme responsible for catalyzing the final reaction of the electron transport system between cytochrome c and oxygen to produce water, a metabolic byproduct. The activity level of cytochrome c oxidase can be used as an indirect indicator of metabolic potential (Ragen et al., 1987; Gnaiger et al., 1998; Gnaiger and Kuznetsov, 2002). Approximately 92% of the activity of cytochrome c oxidase occurs in the inner mitochondrial membrane, with the rest occurring in the outer mitochondrial membrane (Ragen et al., 1987). Cytochrome c oxidase makes up about 21% of the mitochondrial proteins (Ragen et al., 1987). The activity of cytochrome c oxidase is typically measured either through absorbance changes or by the rate of oxygen consumption (Ragen et al., 1987).

Organs with higher metabolic rates may grow out of proportion as body mass increases, and so smaller insects would have a greater amount of highly metabolic tissues per gram body mass as compared to the larger body mass stages. In this study I

investigated histological and gross morphology, total lipid content, and cytochrome oxidase activity levels of developing silkworms (*Bombyx mori*) to determine the changes in structural and biochemical body composition during development. *Bombyx mori* have five morphologically distinct larval stages separated by ecdysis, or molting of the outer cuticle, followed by pre-pupal, pupal and brief adult stages. Body mass changes approximately four-fold during *Bombyx* development, making silkworms an interesting model for observing mass-related oxygen consumption changes on an intraspecific basis (Chapter 2). Oxygen consumption ($\dot{V}O_2$) in *Bombyx mori* viewed across their entire span of development generally follows well documented interspecific trends for oxygen consumption (Chapter 2). However, closer examination indicates a variety of non-standard metabolic allometry patterns within these distinct developmental stages. These complex, stage-dependent relationships most likely reflect major alterations in biochemistry, endocrinology, physiology, morphology, and behavior as well as shifts in the ratio of increasing cell number (hyperplasia) to increasing cell size (hypertrophy). I hypothesized that larval instar I, with the lowest body mass and the highest mass specific $\dot{V}O_2$ (Chapter 2), would have the highest amount of metabolically active tissues per gram of body mass, as indicated by histological, gross morphological, and biochemical measurements.

Materials and Methods

Animal Husbandry

Fertilized eggs of the silkworm, *Bombyx mori*, were obtained from Carolina Biological Supply, Mulberry Farms and Coastal Silkworms. Upon arrival in the laboratory, the eggs were placed in Petri dishes and incubated in a transparent

incubator at a temperature of 25 ± 0.5 °C with a relative humidity of $60 \pm 5\%$. The incubators were illuminated with a LD 12:12 photoperiod. Petri dishes were examined for hatched larvae each morning, since hatching, i.e. eclosion, occurs in the morning due to circadian rhythms of metabolism and eclosion hormone secretion (Fugo et al, 1984). Newly hatched instar I larvae, approximately 3 mm long and 0.0004 g in weight, were transferred into date-labeled Petri dishes. All larval instars were fed an artificial silkworm diet obtained from Carolina Biological Supply in the afternoon and any food remaining in the Petri dishes was removed daily in the evening.

Histological Analyses

Whole animals were fixed with 10% neutral buffered formalin for a minimum 2 h and a maximum of 12 h before dehydration. The specimens were processed (formalin fixed, dehydrated, and paraffin) in a microwave rapid histoprocessor (Milestone RH1), embedded in paraffin blocks (Shandon Histocentre 3 tissue embedder) and cross sections were made between body segments 1-2 and between segments 6-7 with a microtome (Leitz 1512). These sections were chosen to represent the middle/cranial region and the middle/ caudal region. The ribbons were mounted on labeled slides in a 45°C Fisher tissue float bath and allowed to air dry for 24 hours. The tissue was rehydrated and stained with H & E stain. Slides were mounted with Cytoseal XYL and allowed to dry for 24 hours.

Surface area, e.g. total area of cross section, midgut cross section area, was calculated with ImagePro software (Figure 4.1). The midgut cross section included midgut tissue the lumen area was excluded. Total surface area of the larvae was

calculated by multiplying the mean surface area of the cross section (c/s) by the mean body length (assuming the larval body is a tube) for each larval stage.

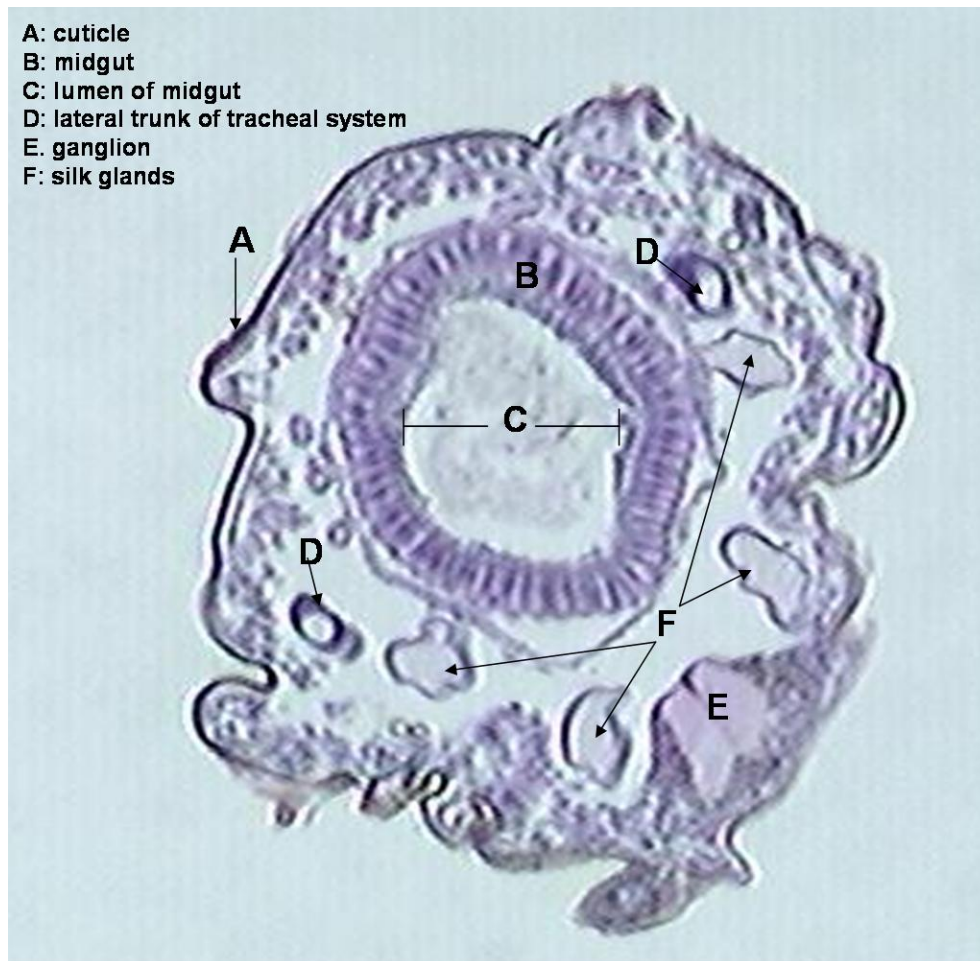


Figure 4.1: Histological light microscopy cross section of a larval instar I *Bombyx mori* with morphology labeled. H & E stained, 100X magnification.

Organ Mass Analyses

Larvae were placed into a 4°C freezer for 30 min for anesthetization. An incision was made ventrally from the mouth to the anus. The midgut, midgut contents, silk glands, and cuticle were dissected free and their wet/dry weights recorded. The remainder of the carcass was weighed to the nearest mg. Dry weights were obtained by placing the wet organs on labeled and pre-weighed weigh boats into a 65°C oven for 72

h. It was determined via a pilot study that there were no differences in dry weights after 48 hours. However, to ensure complete dehydration, 72 h was selected for all measurements. Total dry weight was calculated by adding the component dry weights (midgut, contents, silk glands, cuticle and the remaining carcass).

Body Water Analyses

For each developmental stage, animals (n=7 to 12) were weighed to the nearest mg, placed on labeled, pre-weighed weighing boats, and then dried for 72 h in a 65°C oven. Dehydrated animals were weighed to the nearest mg.

Lipid Analyses

For each developmental stage, (n=7 to 10) silkworms were weighed to the nearest mg and individually placed into 10 ml glass test tubes. Specimens were dehydrated in a 65°C oven for 72 h. The dehydrated animals were macerated with a glass tissue grinder and the resultant material incubated in 4 ml of 2-propanol at 70°C for 30 min. Two ml of chloroform and 1 ml of distilled water were added to each tube and twice mixed with a VOrtex mixer for 2 min. The tubes were tightly capped and held at room temperature for a period of 24 hours. The tubes were centrifuged at 21°C at 600 *rpm* for 12 min. The top layer was collected, washed with KCl and the tubes were mixed with a VOrtex mixer 3 times. The contents of the tubes were allowed to separate into layers and the bottom layer was collected, placed into a pre-weighed glass vial and held at room temperature overnight. After chloroform evaporation under a stream of nitrogen gas, the lipids were measured to the nearest mg.

Mitochondrial Extraction

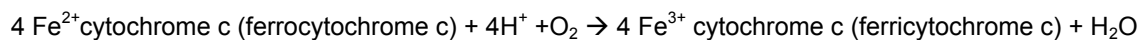
Mitochondria were isolated from whole animal larvae and pupae using a BioChain Mitochondria Isolation Kit for Tissue (Catalog # KC010100). Each animal was weighed and washed twice with 10 ml of PBS. Within 2 h of sampling, tissues were minced with a razor blade inside of glass Petri dishes surrounded by crushed ice. Mitochondrial isolation buffer was added and the solution was placed into a Dounce homogenizer surrounded by crushed ice. The tissue was homogenized for 35 strokes. The homogenate was placed into a 15 ml conical tube and centrifuged at 600g for 10 minutes at -4°C. The pellet was discarded and the solution was re-centrifuged until a clear supernatant was achieved. The supernatant was centrifuged at 12,000g for 15 min at 4°C. The pellet was collected and then re-suspended in 50 μ l of mitochondrial isolation buffer. 100 μ l of mitochondrial storage buffer and 50 μ l of dilution buffer were added to the sample.

Cytochrome Oxidase Analyses

Mitochondria were isolated as described above and utilized in the cytochrome oxidase assay. Cytochrome c oxidase activity was assessed using the BioChain Cytochrome C Oxidase Activity Assay Kit (Catalog # KC310100).

Three trials were performed: a blank, a control, and the mitochondrial extract. Samples were placed in an enzyme dilution buffer and brought to room temperature. Each cuvette had 100 μ l of the sample and 850 μ l enzyme assay buffer, mixed by inversion and a baseline absorbance was recorded. The reduced cytochrome C solution (50 μ l) was added to the cuvette and mixed by inversion. The reaction below was started

and the absorption was read immediately and again at 5 seconds, 15 seconds, 25 seconds, 35 seconds, and at 45 seconds.



The reaction rate was calculated by the following formula:

$$A/\text{min} = (A5s - A45s) \times 60 / 40$$

The activity of the sample was calculated by the following formula:

$$\text{Unit /ml} = \Delta A/\text{min} \times \text{dilution} \times 1 / \text{vol (sample)} \times 21.84$$

where Dilution = dilution factor of the sample, 21.84 = ΔE mM between ferrocytochrome c and ferricytochrome c at 550 nm, and a unit is defined as 1 unit oxidizes 1 μM of ferrocytochrome c per minute at 25C, pH 7.0.

Statistical Analyses

A regression analysis with 95% confidence intervals was performed on the mean total surface area of the cross sections as a function of body mass. A t-test for slopes determined if the resulting slope was significantly different than zero. Independent t-tests were performed between the mean surface area of sections 1-2 and 6-7 for the total cross section and the cross section of the midgut for each developmental stage.

A one-way ANOVA and an SNK analysis were performed on the following means by developmental stage: total area of cross section, area of midgut, the total cross sectional midgut area ratio to total cross sectional area by developmental stage, the wet /dry gross morphological data of the cuticle, silk gland and midgut mass, the body water content, the total fat of wet/dry body mass, and the cytochrome oxidase activity level per gram of body mass. Mean and s.e. are reported. An alpha level of 0.05 was adopted for all statistical decisions.

Results

Histological Analyses

Mean total body cross sectional surface area of larvae ranged from 0.35 mm² in larval instar I to 3324 mm² in the prepupal stage (Figure 4.1). Mean total surface cross sectional area increased as a function of body mass across development ($r^2=0.99$, $b=1.05$, $p<0.001$).

Mean cross sectional area of body segments 1-2 and 6-7 were not significantly different for any stage, suggesting that growth between each of these two instars was by elongation. The combined (section 1-2 and 6-7 for each animal) mean total cross sectional area ranged from 0.11 ± 0.01 mm² for larval instar I to 45.0 ± 1.1 mm² in the prepupal stage (Figure 4.2a). An SNK separated the mean developmental stages into three statistically significant groups: A: larval Instar I, larval Instar II, larval instar III; B: larval instar IV, larval instar V; C. prepupal.

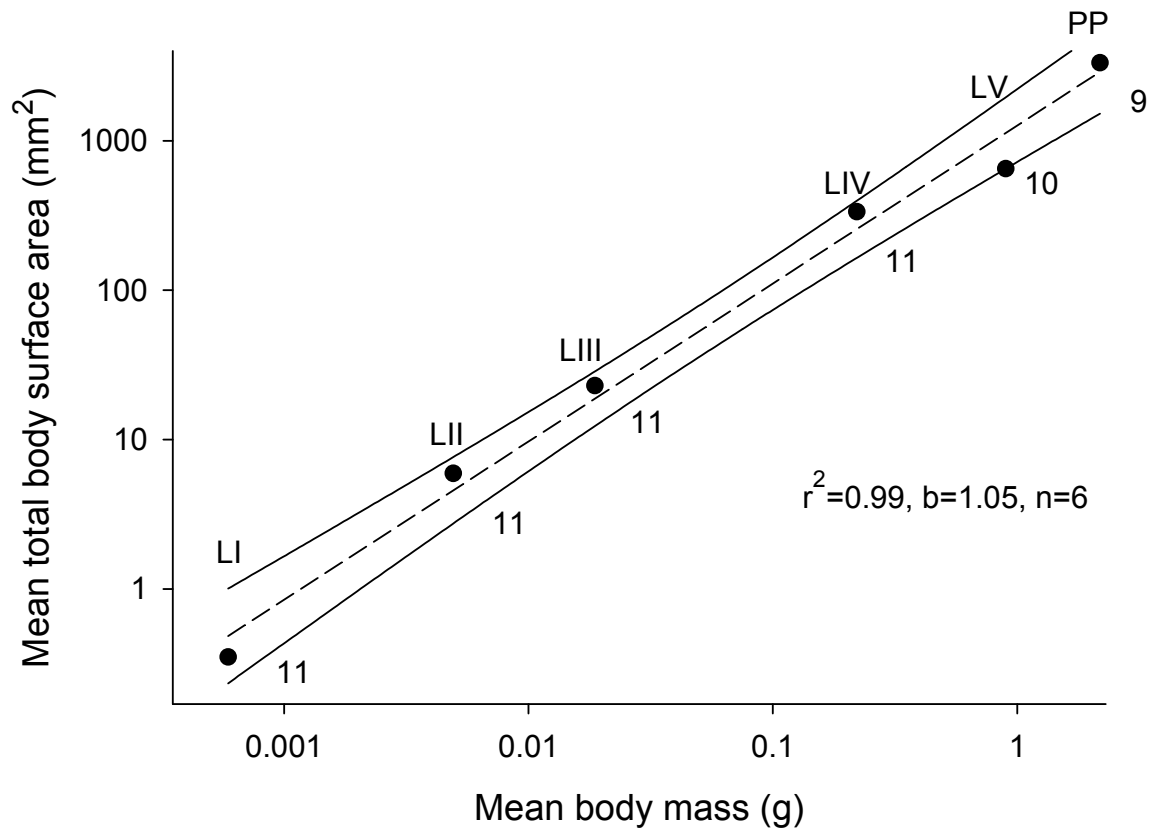


Figure 4.2: Mean total body surface area as a function of body mass of developing *Bombyx mori*. Mean, s.e., developmental stage, regression coefficient, regression lines, 95% confidence intervals and slope are shown.

Mean midgut cross sectional area of body segments 1-2 and 6-7 were not significantly different for any stage (Independent t-test.) Combined mean midgut cross sectional area ranged from $0.030 \pm 0.010 \text{ mm}^2$ for larval instar I to $2.0 \pm 0.2 \text{ mm}^2$ in the prepupal stage (Figure 4.2b). An SNK separated the mean developmental stages into two statistically significant groups: A: larval Instar I, larval Instar II, larval instar III; B: larval instar IV, larval instar V, prepupal.

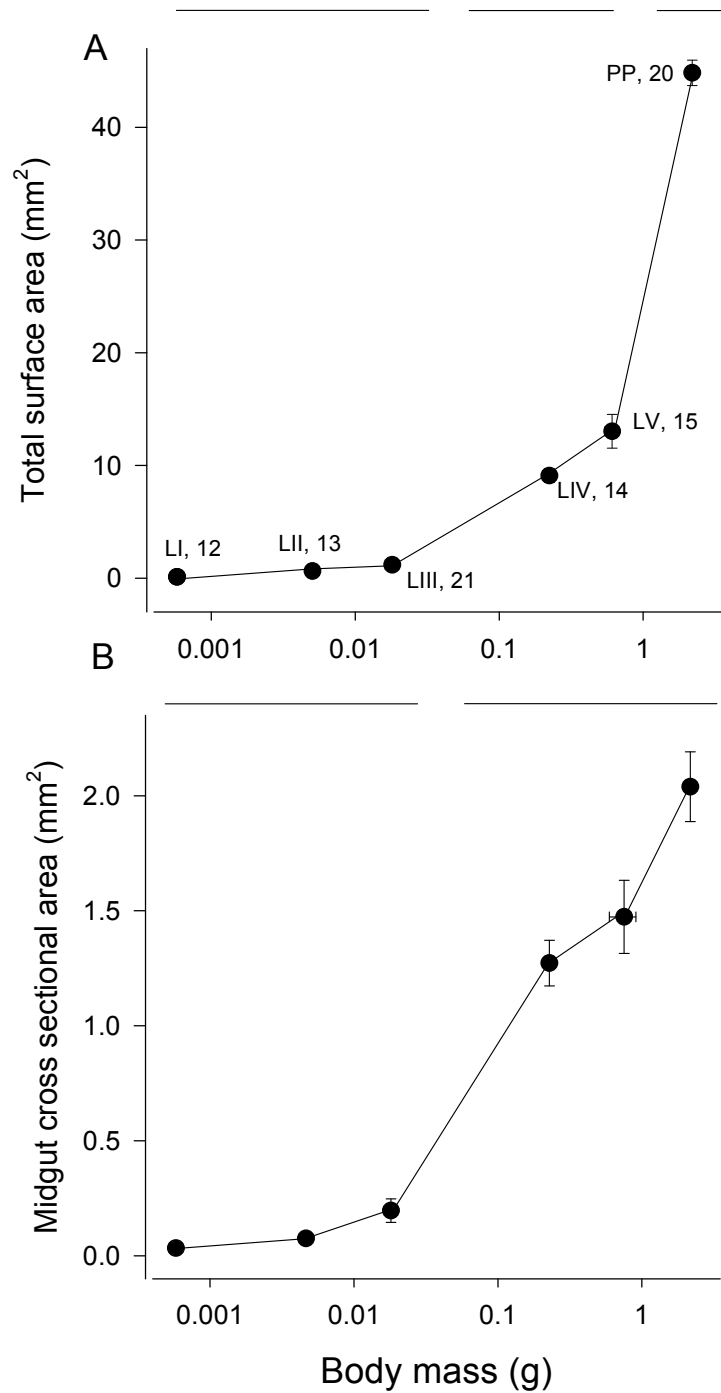


Figure 4.3: Area of cross section of (A) total body and (B) midgut of *Bombyx mori* by body mass. Mean, s.e., n value, SNK groups (horizontal lines) and developmental stages are shown. For panel B, refer to panel A for n values and developmental stage.

Organ Mass Analyses

The mean percentage of cuticle wet mass to total body wet mass ranged from $37.0 \pm 2.0\%$ in larval instar IV to $21.0 \pm 2.0\%$ in the prepupal stage (Figure 4.4a). An SNK separated the mean developmental stages into the following two statistically significant groups: A: larval instar III, larval instar IV, larval instar V; B: prepupal.

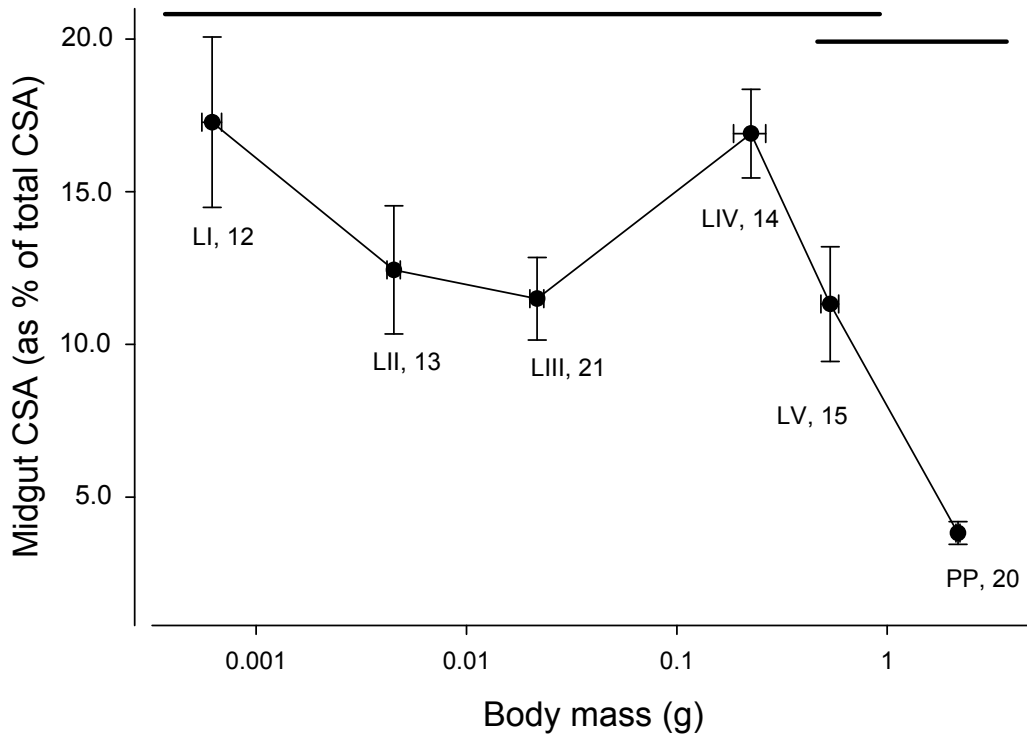


Figure 4.4: Midgut cross sectional area (CSA) as a % total CSA as a function of body mass in *Bombyx mori* by developmental stage. Mean, s.e., n values, and SNK groups (horizontal lines) are shown.

The mean percentage of silk gland wet mass to total body wet mass was $1.0 \pm 0.3\%$ in larval instar III to $12.0 \pm 0.7\%$ in the prepupal stage (Figure 4.4b). An SNK

separated the mean developmental stages into two statistically significant groups: A: larval instar III, larval instar IV, larval instar V; B: prepupal.

The mean percentage of midgut wet mass to total body wet mass was $11.0 \pm 1.0\%$ in larval instar III to $26.0 \pm 0.3\%$ in the prepupal stage (Figure 4.4c). An SNK separated the mean developmental stages into two statistically significant groups: A: larval instar III, larval instar IV; B: larval instar V, prepupal.

The mean percentage of cuticle dry mass to total body wet mass was $44.0 \pm 4.0\%$ in larval instar III to $20.0 \pm 1.0\%$ in the prepupal stage (Figure 4.4d). An SNK separated the mean developmental stages into two statistically significant groups: A: larval instar III, larval instar IV; B: larval instar V, prepupal.

The mean percentage of silk gland dry mass to total body wet mass was 0.80 ± 0.02 in larval instar III to $20.0 \pm 2.0\%$ in the prepupal stage (Figure 4.4e). An SNK separated the mean developmental stages into two statistically significant groups: A: larval instar III, larval instar IV, larval instar V; B: prepupal.

The mean percentage of midgut dry mass to total body wet mass was $7.0 \pm 0.7\%$ in larval instar V to $17.0 \pm 1.0\%$ in the prepupal stage (Figure 4.4f). An SNK separated the developmental stages into three statistically significant groups: A: larval instar III, larval instar IV; B: larval instar V; C: prepupal.

Body Water Analyses

The mean proportion of total body water (as a percentage of total body mass) ranged from $67.0 \pm 2.0\%$ in adults to $86.0 \pm 3.0\%$ in larval instar IV (Figure 4.5). An SNK separated the mean developmental stages into three statistically significant

groups: A: larval Instar I, pupal, adults; B: larval instar II, larval Instar III, larval instar IV, larval instar V; C: prepupal.

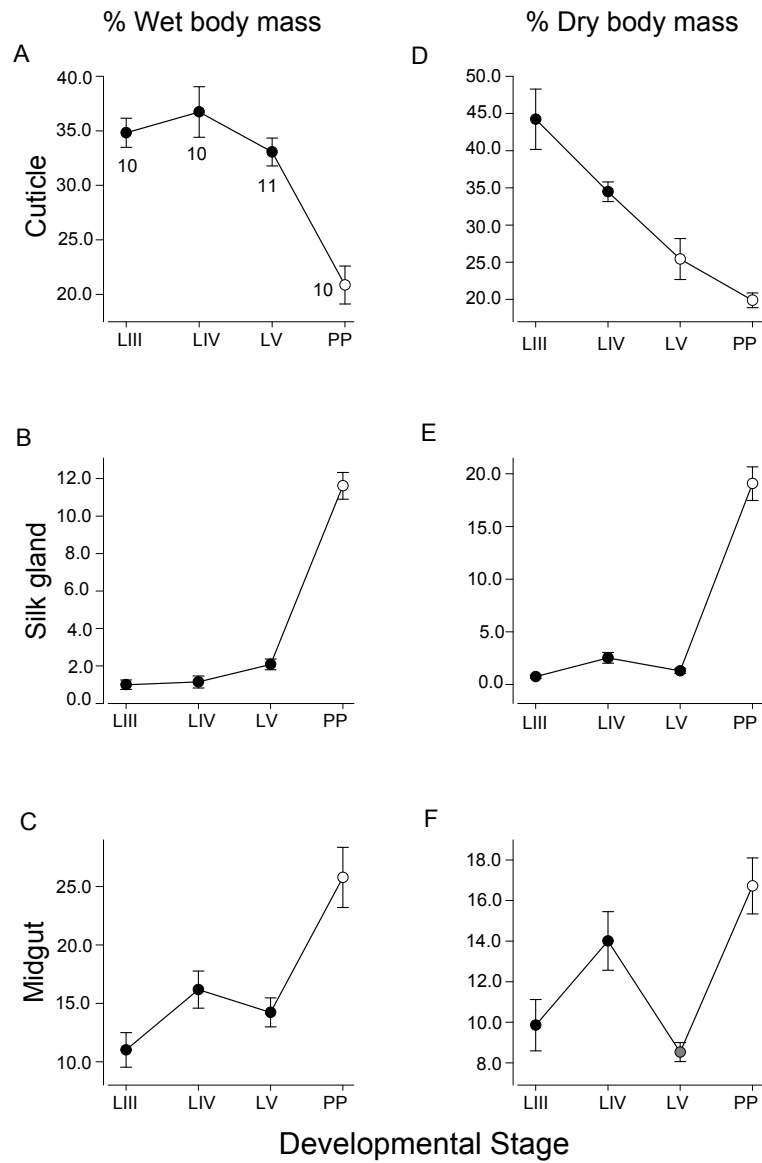


Figure 4.5: Mean ratio of (A / D) cuticle, (B / E) silk gland and (C / F) midgut to total body mass for each developmental stage of *Bombyx mori*. Mean, s.e., n values are shown. SNK groups are differentiated by different symbols.

Lipid Analyses

Mean percentage of body fat of total wet body mass ranged from $0.90 \pm 0.04\%$ in larval instar V to $10.0 \pm 0.2\%$ in adults (Figure 4.6a). An SNK separated the mean developmental stages into three statistically significant groups: A: larval instar I, adults; B: larval instar II, larval instar III, prepupal and pupal; C: larval instar IV, larval instar V.

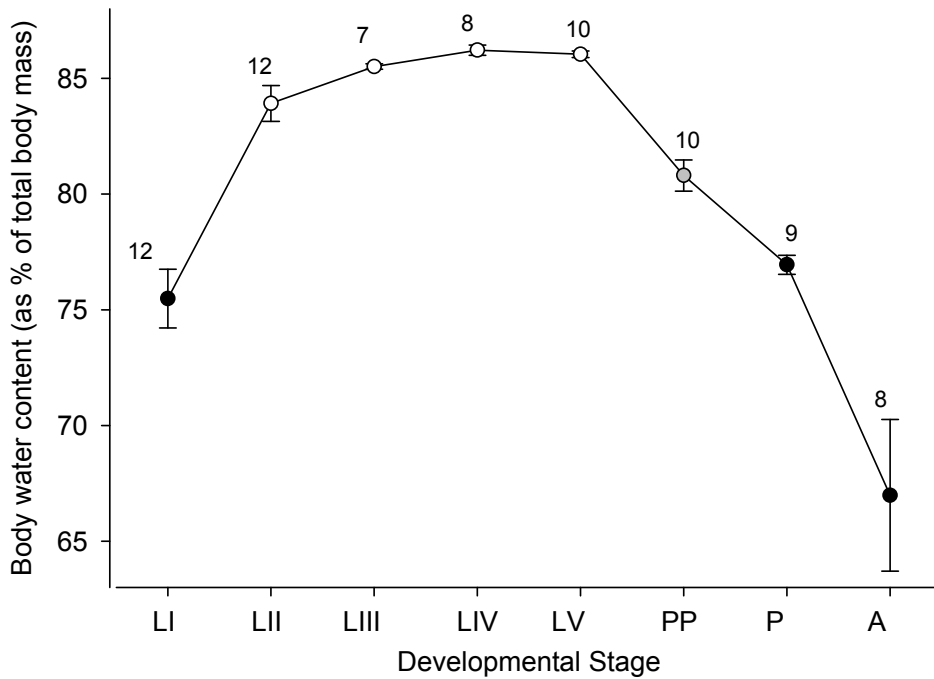


Figure 4.6: Body water content (as a % of total body mass) in *Bombyx mori* as a function of developmental stage. Mean, s.e., n values are shown. SNK groups are differentiated by different symbols.

Mean percentage of body fat of total dry body mass ranged from $6.0 \pm 0.3\%$ in larval instar V to $36.0 \pm 0.3\%$ in larval instar I (Figure 4.6b). An SNK separated the mean developmental stages into four statistically significant groups: A: larval instar I, adults; B: larval instar II, larval instar III, prepupal; C: larval instar IV, larval instar V. D: pupal.

Cytochrome Oxidase Analyses

Mean cytochrome oxidase activity ranged from $0.21 \pm 0.02 \text{ unit ml}^{-1} \text{ g}^{-1}$ in the prepupal stage to $2.80 \pm 0.41 \text{ unit ml}^{-1} \text{ g}^{-1}$ in larval instar I (Figure 4.7). The developmental stages were separated into 4 statistically significant groups as follows: A: larval instar I, larval Instar II, larval instar III; B: larval instar IV; C: larval instar V, pupal; D: prepupal, adults.

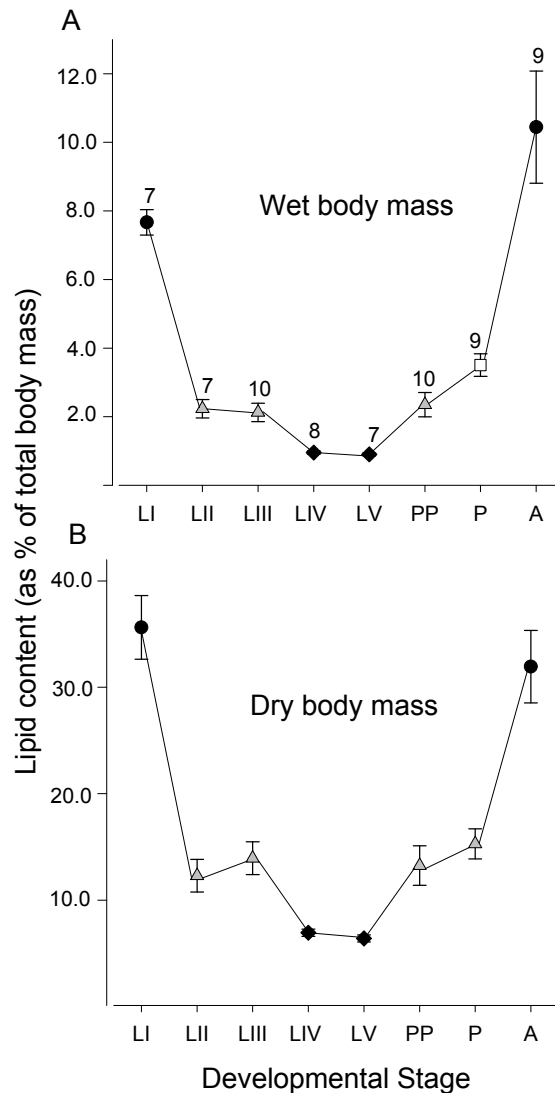


Figure 4.7: Lipid content as a % of (A) wet body mass and (B) dry body mass in *Bombyx mori* by developmental stage. Mean, s.e. and n values are shown. SNK groups are differentiated by different symbols.

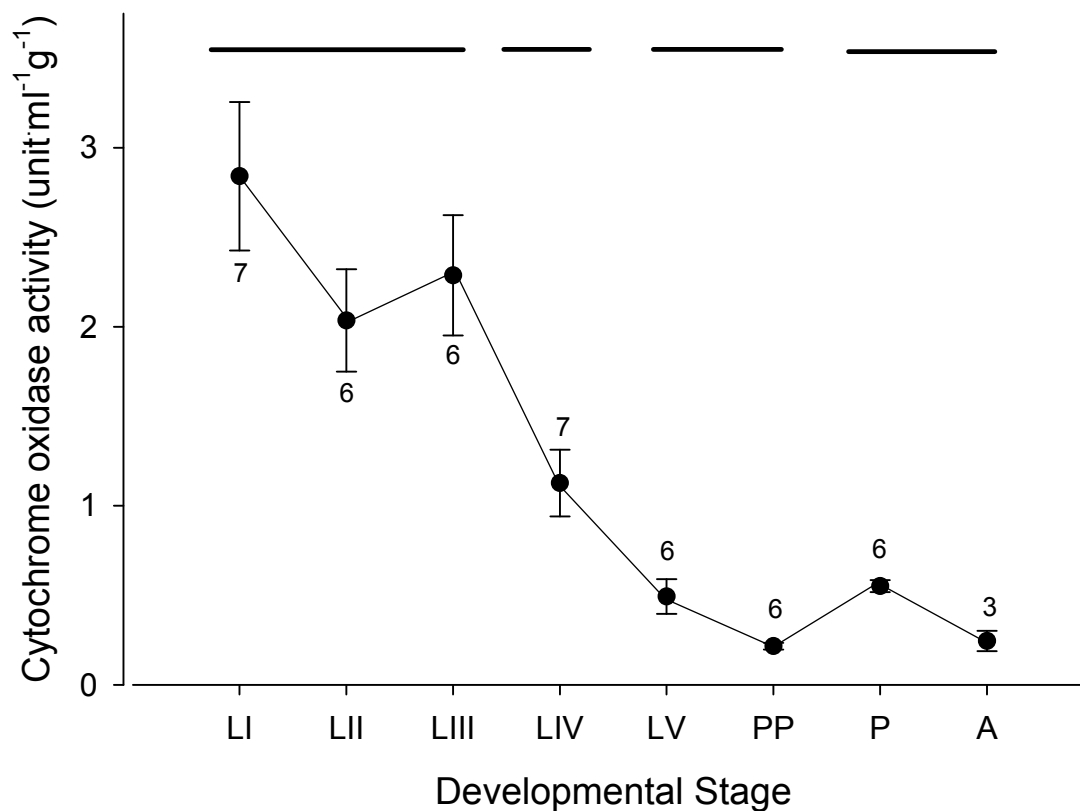


Figure 4.8: Cytochrome oxidase activity per gram of body mass by developmental stage of *Bombyx mori* silkworms. Mean, s.e. n values and SNK groups are shown (by horizontal lines).

Discussion

Histological Analyses

Mean total body surface area, ranging from 0.35 mm² in larval instar I to 3324 mm² in the prepupal stage, was consistent with data on the tobacco horn moth (*Manduca sexta*) which ranged from 25mm² in larval instar I to 5000 mm² in larval instar V (Baldwin and Hakim, 1991). The poikilothermic cutaneous surface area is not related

to metabolic rate as it may be in homeothermic animals as the heat dissipated from the cuticle does not affect the metabolic rate.

The mean midgut cross sectional area (CSA) increased throughout development, but when viewed as a percentage of the total CSA as a function of body mass, there was an inverse relationship and the prepupae had the lowest percentage. Older larvae have a smaller midgut CSA as a function of body mass; however, these later stages of development have increased effectiveness in digestion and absorption than younger larvae (Gibellato and Chamberlin, 1994). Interestingly, this increase in efficiency of the midgut epithelium does not contribute to an increase in the $\dot{V}O_2$ (Gibellato and Chamberlin, 1994).

Organ Mass Analyses

The stages with the highest mass-specific $\dot{V}O_2$ (larval instars I – III) had the highest proportion of cuticle compared to other stages (Chapter 2). Therefore, since the cuticle is an organ with comparatively low metabolic potential, the cuticle is unlikely to be a factor contributing to changes in mass specific $\dot{V}O_2$ across development.

The silk glands of *Bombyx mori* should also be considered a highly metabolically active organ due to the energy-dependent synthesis and secretion of silk proteins (Matsuura et al., 1968; Morimoto et al., 1968; Tashiro et al, 1968; Sasaki and Tashiro, 1976). The stages with the highest mass specific $\dot{V}O_2$ (larval instars I – III) had the lowest percentage of the silk glands of total body mass as compared to the other stages. Silk glands are utilized throughout development and the mass of the silk gland rapidly reaches its peak by the prepupal stage and is followed by a rapid catabolism during metamorphosis (Matsuura, et al., 1968). One of the stages with the lowest mass-

specific $\dot{V}O_2$, the prepupae, had the significantly highest percentage of silk glands of total body mass when compared to the other stages. Therefore, since the silk gland is an organ with high metabolic potential, the silk glands must not be a factor contributing to changes in mass specific $\dot{V}O_2$ across development.

The stages with the highest mass specific $\dot{V}O_2$ (larval instars I – III) had the lowest percentage of the midgut of total body mass as compared to the other stages. The midgut mass of the prepupae had a significantly higher percentage to total body mass as compared to other stages. In contrast, the prepupae had the lowest ratio of the cross section surface area of the midgut to the total cross section area. Therefore, the midgut must not occupy as much volume in the body, but rather the tissue must increase in density and mass. Possibly the mechanisms increasing the efficiency of the midgut tissue contribute to this increase in mass. There is a surge of ecdysone at the onset of the pupal stage and mitochondria decrease in the malphigian tubule microvilli in response to this hormone and further decrease during metamorphosis (Bradley, 1984). Therefore, the midgut might have a larger mass, but the onset of metamorphosis could cause the number of mitochondria to decrease and lower the metabolic activity of the tissue on a per gram basis (Chamberlin, 2006).

Tissue Water Composition

Overall, the findings for body water content of *Bombyx mori* are in agreement with data for insects, which ranges from 60-90% of wet mass (Gordon, 1968). Larval instar I hatch with the lowest body surface area to Volume ratio of the developing larval stages. These animals utilize only yolk for energy during incubation and consume their first meal within 24 hours after hatching. In *Bombyx mori*, water is primarily obtained

from food so it is understandable that the stages with either minimal (larval instar I) or absent (prepupal to adult) consumption of food (i.e. water) would have the lowest body percentages of water compared to other stages. The lowest body percentages of water were larval instar I, pupae and the adults.

The prepupal stage was significantly separated from the earlier stages with a decrease in water body mass. During the terminal end of the final instar, the insect consumes as much food as possible for storage, but once the food consumption stops in the prepupal stage, water loss begins.

Lipid Analyses

Fats, as well as glycogen, increase in the insect body up until metamorphosis when they then start to decline (Tojo et al., 1978). When considered on a per gram of body mass basis, in *Bombyx mori* larval instar I and the adults had the highest percentage of total body lipid mass. These stages also had the lowest percentage of body water and this might contribute to the elevation of the lipid mass. Larval instar I leave a physiological non-feeding period of embryogenesis upon hatching and the adults are at the terminal non-feeding period. This may explain why the tissue body water of these two stages are statistically identical.

As expected, the lowest lipid contents was in the Larval instar IV and V stages as they are undergoing routine feeding and do not possess an increased fat storage for long-term survival. It isn't until the last days of larval instar V when the larvae commence the intensive feeding stage for energy storage.

The lipid contents of the present study differ slightly from the overall lipid contents reported for the cecropia moth (*Hyalophora cecropia*) - 9.2% over development

and 16.9% for adults (Domroese and Gilbert, 1964). These lipid content differences might be attributed to species or by gender selection, since lipid content of the *Hyalophora cecropia* silk moth is dependent on gender (Domroese and Gilbert, 1964). However, my findings are in agreement with data for *Bombyx mori* and other Lepidopterans (Fast, 1964; Prasad et al., 1986).

Cytochrome Oxidase Analyses

Cytochrome oxidase activity increases throughout development of flies (*Lucilia*) considered on a whole animal basis (D'Costa and Birt, 1966). On a mass-specific basis, cytochrome activity of course decreases over development. These relationships follow the same pattern as $\dot{V}O_2$ as a function of body mass.

The stages with the highest mass specific $\dot{V}O_2$ (Larval instars I – III) had the highest cytochrome oxidase activity levels per gram of body mass. The lowest activity levels per gram were in the prepupal and in the adult stages. The electron transport chain (ETC) complex (including cytochrome oxidase) in the midgut of lepidopterans decline in activity throughout development and the amount of cytochrome oxidase activity declines as programmed cell death occurs during metamorphosis (Chamberlin, 2006). However, cytochrome oxidase might not be the sole predictor of metabolic activity. Cytochrome oxidase is found in excess in mitochondria thereby making oxygen a possible limiting factor for the activity of cytochrome oxidase (Chamberlin, 2004).

Conclusion

The data presented above refute the notion that the higher metabolic rate organs grow out of proportion with increasing body mass, and that the smaller insects would have a greater amount of highly metabolic tissues per gram body mass as

compared to the larger body mass stages. However, the smaller body mass instars (larval instar I-III) had the highest amount of mitochondrial cytochrome oxidase activity per gram of body mass. This provides evidence that there are more metabolically active mitochondria per gram in those developing larvae with a smaller body mass. Therefore, the amount of activity of mitochondria per unit mass of the highly metabolically active organs might contribute to the increased mass-specific $\dot{V}O_2$ of the younger larvae.

CHAPTER 5

CONCLUSION

The silkworm, *Bombyx mori*, shows complex changes in body mass during its life cycle. From the time instar I larva hatches, there is a routine gain in body mass through the larval stages and once these insects enter metamorphosis, the energy stored during the prepupal stage will be the sole energy available for the remainder of the lifecycle. Not surprisingly, then, once the insect reaches a maximum weight in the prepupal stage, the body mass decreases with age until subsequent death.

Changes in whole animal and mass-specific $\dot{V}O_2$ during development in the silk worm were in agreement with previous literature on oxygen consumption of developing insects and generally aligned with the interspecific “mouse to elephant curve”. However, regression analysis of oxygen consumption as a function of body mass of individual developmental stages revealed relationships that were significantly different from the overall regression throughout development.

In addition to showing interesting intraspecific differences in the allometry of metabolic rate, the present study also indicates that the overall developmental slope should not be used for the comparison of growing animals that are undergoing complex developmental stages. Interspecific comparison of developing animals should not be made unless the physiological states and structures are analogous. This type of data should be presented as individual developmental stages of whole animal $\dot{V}O_2$ as a function of body mass - which is the most appropriate method of presenting intraspecific metabolic allometric relationships.

There were four methods of presentation of intraspecific allometric $\dot{V}O_2$ relationships, in this study: whole animal $\dot{V}O_2$ as a function of body mass, mass-specific $\dot{V}O_2$ as a function of body mass, whole animal $\dot{V}O_2$ as a function of surface area, and mass-specific $\dot{V}O_2$ as a function of surface area. $\dot{V}O_2$ as a function of surface area is an appropriate method of presenting intraspecific allometric relationships. However, based on regression analysis, the most appropriate method of presentation of intraspecific allometric relationships is $\dot{V}O_2$ as a function of body mass ($r^2 = 0.96$). Whole animal $\dot{V}O_2$ is a more accurate method of describing intraspecific allometric relationships than mass-specific $\dot{V}O_2$ judging by the statistical analyses presented in Chapter 2.

Whole animal $\dot{V}O_2$ was greater in females during the prepupal stage, when females are typically larger than males. Therefore, the larger $\dot{V}O_2$ would not necessarily be attributed to gender differences, but rather to differences in body mass. In adults, however, the whole animal $\dot{V}O_2$ rates were larger for the males, despite still being the smaller of the two sexes. The difference in adult $\dot{V}O_2$ can likely be attributed to the elevated activity possibly due to the stimulation of pheromone receptors in males.

At the end of the sedentary physiological state of the pupal stage, the adult moths emerge for the sole function of reproduction. The female moths maintain minimal activity whereas male moths have elevated activity by observation, possibly due to numerous sensilla located on their antennae that are stimulated by the female pheromones – bombykal and bombykol (Sakuri, et al., 2004). One male in the preset study exhibited a $\dot{V}O_2$ of $15.12 \text{ ml O}_2 \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$ during a measurement period, which is 18 times higher than average for females and approaches the metabolic rate of an active hummingbird ($15.58 \text{ ml CO}_2 \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$) (Powers and Conley, 1994). This raises an

interesting question for male adult *Bombyx mori*'s maximum aerobic metabolic scope and energy store utilization, for example, is the tracheal system modified in the males?

Based on RQ values, larval instar I of *Bombyx mori* primarily metabolized carbohydrates from embryogenesis. A switch to fat metabolism occurred during the next three larval stages. This might be attributed to food deprivation the twelve hours prior to oxygen consumption measurements. Prepupal RQ increased above 1.0 during preparation processes for metamorphosis. An RQ above 1.0 can be an indicator for lipogenesis (Kleiber, 1975). During the prepupal stage, the insect has undergone an intensive feeding stage and synthesizes fat stores for the remainder of its life cycle. The RQ of 0.86 during metamorphosis in *Bombyx mori* in the present study could either be explained by protein metabolism or a mixture of carbohydrate and lipid metabolism as metamorphosing pupae undergo histolysis and histogenesis simultaneously.

I questioned the validity of measuring the $\dot{V}O_2$ of pupa in intact cocoons as opposed to naked pupa. Since there were no significant differences between $\dot{V}O_2$ or body mass loss (via water loss) between intact pupa and naked pupa, measuring $\dot{V}O_2$ of intact pupa should be considered a legitimate method. When measuring $\dot{V}O_2$ of intact cocoons, the cocoon was not found to be a short-term limiting factor for either $\dot{V}O_2$ or for the rate of mass loss. The cocoon thus appears to have the function of providing mechanical protection during metamorphosis. I also found that reporting $\dot{V}O_2$ values of metamorphosing pupae utilizing the entire body mass (living and non-living) is an appropriate method.

Midgut surface area and percentage of midgut mass (of total body mass) decreases with development (Chapter 4), the percentage of cuticle mass (of total body

mass) decreases with development, and the percentage of silk gland mass (of total body mass) increases with development. These data do not support the assumption that the higher metabolic organs grow out of proportion and that the smaller insects would have a greater amount of highly metabolic tissues per gram body mass as compared to the larger body mass stages. Total lipid and body water mass were also not shown to contribute to changes in $\dot{V}O_2$.

However, the stages with the highest mass-specific $\dot{V}O_2$ (larval instars I – III) had the highest percentage of cytochrome oxidase activity levels per g of body mass. The lowest activity levels of cytochrome oxidase per g were in the prepupal and adult stages. This provides evidence that there is a greater percentage of metabolic activity in the mitochondria per g in the insects with a smaller body mass. In mammals, larger animals have smaller surface areas of their inner mitochondrial membranes in liver tissue (Agutter and Wheatley, 2004) and this could be one possible explanation for *Bombyx* having less mitochondrial activity in the larger animals. Therefore, the activity of the mitochondria per unit mass of the highly metabolically active organs might attribute to the increased mass-specific $\dot{V}O_2$ of the smaller body mass animals.

These observations collectively support the idea that intraspecific allometric relationships of metabolism generally follow the trend of the interspecific allometric relationships of adult metazoans, but also have significantly different relationships within individual instars. Further investigations should be made to determine when the developing larvae switch from hyperplastic growth to hypertrophic growth to ascertain whether these processes have an influence over the changes in the rates of $\dot{V}O_2$ during development. Therefore, I support my initial hypothesis of the differences in intraspecific

developmental metabolic allometric patterns by lifecycle stage over the course of development in *Bombyx mori*.

The marked differences in behavior, metabolic and physiologic processes, and body composition between developmental stages during larval development and metamorphosis in insects result in additional complexity to the differing demands for energy supply associated with large increases in body mass.

In summary, an allometric analysis of intraspecific developmental relationships of metabolism as a function of body mass should include an analysis of individual stages of development as well as an analysis of development as a whole to gain a comprehensive understanding of the complexity of allometry of the developing animal such as the silkworm.

REFERENCES

- Agutter, P. S. and Wheatley, D. N. (2004). Metabolic scaling: consensus or controversy? *Theor. Biol. Med. Model.* 16, 1-13.
- Allen, W. V. (1976). Biochemical aspects of lipid storage and utilization in animals. *Amer. Zool.* 16, 631-647.
- Asakura, T., Umemura, K., Nakazawa, Y., Hirose, H., Higham, J., and Knight, D. (2006). Some observations on the structure and function of the spinning apparatus in the silkworm *Bombyx mori*. *Biomacromolecules*. 8: 175-181.
- Baldwin, K.M., and Hakim, R.S. (1991). Growth and differentiation of the larval midgut epithelium during molting in the moth, *Manduca sexta*. *Tissue and Cell*. 23(3): 411-422.
- Batterham, A.M., Vanderburgh, P.M., Mahar, M.T., and Jackson, A.S. (1999). Modeling the influence of body size on $VO_{2\text{ peak}}$: effects of model choice and body composition. *J Appl. Physiol.* 87(4): 1317-1325.
- Bennett, V.A., Kukal, O. and Lee, R.L. (1999). Metabolic opportunists: feeding and temperature influence the rate and pattern of respiration in the high arctic woollybear caterpillar *Gynaephora grownlandica* (Lymantriidae). *J. Exp. Biol.* 202(1): 47-53.
- Bradley, T. J. (1984). Mitochondrial placement and function in insect ion-transporting cells. *Amer. Zool.* 24, 157-167.
- Brody, S. (1945). Bioenergetics and growth. Reinhold Publishing. New York.
- Brown, J.H., Gillooly, J.F., Allen, A.P., Savage, V.M., and West, G.B. (2004). Toward a metabolic theory of ecology. *Ecology*. 85:1771-1789.
- Burggren, W. W. (2005). Developing animals flout prominent assumptions of physiological ecology. *Comp. Biochem. Physiol.* 141(A): 430-439.
- Burggren, W.W. and Monticino, M.G. (2005.) Assessing physiological complexity. *J. Exp. Biol.* 208:3221-3232
- Burness, G. P. (2002). Elephants, mice and red herrings. *Science*. 296(5571): 1245-1247.
- CanaVOso, L. E., Yun, H. K., Jouni, Z. E. and Wells, M. A. (2004). Lipid transfer particle mediates the delivery of diacylglycerol from lipophorin to fat body in larval *Manduca sexta*. *J. Lip. Res.* 45:56-465.

- CanaVOso, L. E., Jouni, Z. E., Karnas, K. J., Pennington, J. E., and Wells, M. A. (2001). Fat metabolism in insects. *Annu. Rev. Nutr.* 21:23-46.
- Chamberlin, M.E. (2004.) Control of oxidative phosphorylation during insect metamorphosis. *Amer.J. Phys. Reg. Int. Comp. Phys.* 287: 314-321.
- Chamberlin, M.E. (2006). Changes in mitochondrial electron transport chain activity during insect metamorphosis. *Am. J. Physiol. Reg. Integr. Comp. Physiol.* [epub ahead of print.]
- Chown, S. L. and Davis, A.L.V. (2003). Discontinuous gas exchange and the significance of respiratory water loss in scarabaeine beetles. *J. Exp.Biol.* 206: 3547-3556.
- Couture, P., and Hulbert, A.J. (1995). Relationship between body mass, tissue metabolic rate, and sodium pump activity in mammalian liver and kidney. *Am J Physiol Regul Integr Comp Physiol* . 95: R641-R650.
- Darveau, C.A., Suarez, R.K., Andrews, R.D., Hochachka, P.W. (2002). Allometric cascade as a unifying principle of body mass effects on metabolism. *Nature*. 417: 166-170.
- D'Costa, M.A., and Birt, L.M. (1966). Changes in the lipid content during the metamorphosis of the blowfly, *Lucilia*. *J. Ins. Physiol.* 12:1377-1394.
- Domroese, K. A. and Gilbert, L. I. (1964). The role of lipid in adult development and flight muscle metabolism in *Hyalophora cecropia*. *J. Exp. Biol.* 41:573-590.
- Downer, R. G. H., and Matthews, J. R. (1976). Patterns of lipid distribution and utilization in insects. *Amer. Zool.* 16:733-745.
- Economo, E. P., Kerkhoff, A. J. and Enquist, B. (2005). Allometric growth, life-history invariants and population energetics. *Ecol. Lettters* 8:353-360.
- Edwards, J. (1953). Insect physiology. Wiley Publishing, New York.
- Else, P.L. and Hulbert, A.J. (1981). Comparison of the “mammal machine” and the “reptile machine”: Energy Production. *Amer. J. Physiol. Reg. Int. Comp .Physiol.* 240:3-9.
- Emlen, D. J. and Allen, C. E. (2004). Genotype to phenotype: physiological control of trait size and scaling in insects. *Integr. Comp. Biol.* 43:617-634.
- Fast, P. (1964.) Insect Lipids: A review. *Mem. Ent. Soc. Can.* 37: 3-50.

- Feldman, H. A. and McMahon, T.A. (1983). The $\frac{3}{4}$ mass exponent for energy metabolism is not a statistical artifact. *Resp.Physiol.* 52:149-163.
- Fernando-Warnakulasuriya, G. J. P., Tsuchida, K. and Wells, M. A. (1988). Effect of dietary lipid content on lipid transport and storage during larval development of *Manduca sexta*. *Insect Biochem.* 18(2):211-214.
- Fink, D. E. (1925). Metabolism during embryonic and metamorphic development of insects. *J. Gen. Physiol.* 527-543.
- Frankino, W. A., Zwaan, B. J., Stern, D. L. and Brakefield, P. M. (2005). Natural selection and developmental constraints in the eVOLution of allometries. *Science.* 307(5710):718-720.
- Frew, J. G. H. (1927). Studies in the metabolism of insect metamorphosis. *J. Exp. Biol.* VI(3):206-217.
- Fugo, H., Hitoshi S., Hiromichi N., and Suzuki, A. (1985). Eclosion hormone activity in developing embryos of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 31(4):293-298.
- Ganga, G. (2003). Comprehensive sericulture VOI 2: Silkworm rearing and silk reeling. Science Publishers Inc., Plymouth UK.
- Gauthier, N., Mandon, N. Renault, S., and Benedet, F. (2004). The *Acrolepiopsis assectella* silk cocoon: Kairomonal function and chemical characterization. *J. Ins. Physiol.* 50:1065-1074.
- German, R. Z. and Meyers, L. L. (1989). The role of time and size in ontogenetic allometry: I. Review. *Growth, development and aging.* 53:107-115.
- Gibbs, A. G. and Johnson, R. A. (2004). The role of discontinuous gas exchange in insects: the chthonic hypotheses does not hold water. *J. Exp. Biol.* 207:3477-3482.
- Gibellato, C. M. and Chamberlin, M. E. (1994). Midgut metabolism in different instars of the tobacco hornworm (*Manduca sexta*.) *J. Exp. Zoolog.* 270:405-409.
- Gnaiger, E., Lassnig, B., Kuznetsov, A., Rieger, G., and Margreiter, R. (1998). Mitochondrial oxygen affinity, respiratory flux control and excess capacity of cytochrome c oxidase. *J. Exp. Biol.* 201:1129-1139.
- Gnaiger, E., Kuznetsov, A.V. (2002). Mitochondrial respiration at low levels of oxygen and cytochrome c. *Biochem. Soc. Trans.* 30(2):411-417.

- Gray, Emilie M. and Timothy J. Bradley. (2006). Evidence from mosquitoes suggest that cyclic gas exchange and discontinuous gas exchange are two manifestations of a single respiratory pattern. *J. Exp. Biol.* 209:1603-1611.
- Greenlee, K.J., and Harrison, J.F. (2004). Development of respiratory function in the American locust *Schistocerca americana* II: Within instar effects. *J. Exp. Biol.* 207:509-507.
- Gordon, H. T. (1968). Quantitative aspects of insect nutrition. *Am. Zoolog.* 8:131-138.
- Grant, J.B. (2006). Diversification of gut morphology in caterpillars is associated with defensive behavior. *J. Exp. Biol.* 209:3018-3024.
- Hakimi, O., Knight, D.P., Knight, M.M., Grahn, M.F., and Vadgama, P. (2006). Ultrastructure of insect and spider cocoon silks. *Biomacromolecules.* 7:2901-2908.
- Hetz, S.K. and T.J. Bradley. (2005). Insects breathe discontinuously to aVOid oxygen toxicity. *Nature.* 433:516-519
- Heusner, A. A. (1982). Energy Metabolism and Body Size. I. Is the 0.75 Mass exponenet of Kleiber's equation a statistical artifact? *Resp. Phys.* 48:1-12.
- Heyland, A. and Moroz, L.L. (2006). Signaling mechanisms underlying metamorphic transitions in animals. *J. Intgr.Comp. Biol.* 46:743-759.
- Hochachka, P.N., Darveau, C.A., Andrews, R.D., and Suarez, R.K. (2003). Allometric cascade: A model for resolving body mass effects on metabolism. *Comp. Biochem. Physiol.* 134(A): 675-691.
- Hostetler, M.E., Anderson, J.F., and Lanciani, C.A. (1994). *Florida Entomol.* 77(2): 288-291.
- Hulbert, A.J., and Else, P.L. (2005). Membranes and the setting of the energy demand. *J. Exp. Biol.* 208:1593-1599.
- Hunt Von Herbing, I. (2006). The physiological basis for metabolic scaling in animals: a developing perspective. *Comp. Dev. Phys.* 6:83-95.
- Jouni, Z. E. and Wells, M. A. (1996). Purification and partial characterization of a lutein-binding protein from the midgut of the silkworm *Bombyx mori*. *J. Biol. Chem.* 271(25):14722-14726.
- Kanzaki, R., Soo, K., Seki, Y., and Wada, S. (2003). Projections to higher olfactory centers from subdivisions of the antennal lobe macroglomerular complex of the male silkmoth. *Chem. Senses.* 28:113-130.

- Kazuyuki U., Hiroyuki, A., Kiyoaki K., Tomoko M. and Makoto K. (2005). Excitatory neural control of posterograde heartbeat by the frontal ganglion in the last instar larva of a lepidopteran, *Bombyx mori*. *J. Comp. Physiol.* 192(2):1432-1451.
- Kleiber, M. Body Size and Metabolism. (1932). *Hilgardia: A journal of agricultural science. California Agricultural Experiment Station.* 6(11):315-349.
- Kleiber, M. (1975). Fire of Life: An introduction to animal energetics. Robert E. Krieger Publishing Company. Huntington, New York.
- Klingenberg, C. P. (1998). Heterochrony and allometry: the analysis of eVOLutionary change in ontogeny. *Biol. Rev.* 73:79-123.
- Kodric-Brown, A., Sibly, R.M. and Brown, J.H. (2006). The allometry of ornaments and weapons. *PNAS.* 103(23):8733-8738.
- Kozlowski, J., M. Konarzewski, and A. T. Gawelczyk. (2003). Cell size as a link between noncoding DNA and metabolic rate scaling. *PNAS.* 100:14080-14085.
- Kozlowski, J. and Konarzewski, M. (2004). Is West, Brown and Enquist's model of allometric scaling mathematically correct and biologically relevant? *Funct. Ecol.* 18:283-289.
- Law, J.H., and Wells, M.A. (1989). Insects as biochemical models. *J. Biol. Chem.* 264(28):16335-16338.
- Levy, Robert I. and H. A. Schneiderman (1966). Discontinuous respiration in insects – IV. Changes in intratracheal pressure during the respiratory cycle of silkworm pupae. *J. Insect Physiol.* 12:465-492.
- Lighton, J. R.B. (1988). Discontinuous CO₂ emission in a small insect, the formicine ant *Camponotus vicinus*. *J. Exp. Biol.* 134:363-376.
- Lighton, John R. B. (1991). Ventilation in namib desert tenebrionid beetles: mass scaling and evidence of a novel quantized flutter-phase. *J. Exp. Biol.* 159: 249-268.
- Lighton, J. R.B. and Lovegrove, B. G. (1990.) A temperature induced switch from diffusive to convective ventilation in the honeybee. *J. Exp. Biol.* 154:509-516.
- Lighton, J. R. B., and Ottesen, E. A. (2005). To DCG or not to DCG: oxygen guarding in the termite *Zootermopsis nevadensis* (Isoptera: termopsidae.) 208:4671-4678.

- Lighton, J. R. B., Schilman, P.E. and Holway, D.A. (2004). The hyperoxic switch: assessing respiratory water loss rates in tracheate arthropods with continuous gas exchange. *J. Exp. Biol.* 207:4463-4471.
- Mandel, L. J., Riddle, T. G., and Storey, J. M. (1980). Role of ATP in respiratory control and active transport in tobacco hornworm midgut. *Am. J. Physiol. Cell. Physiol.* 238:C10-C14.
- Mandel L.J., Moffett D.F., and Jobsis F.F. (1975). Redox state of respiratory chain enzymes and potassium transport in silkworm mid-gut. *Biochem Biophys Acta.* 408(2):123-34.
- Marais, E. and Chown, S.L. (2003). Repeatability of standard metabolic rate and gas exchange characteristics in a highly variable cockroach, *Perisphaeria sp.* *J. Exp. Biol.* 206:4565-4574.
- Matsuura, S., Morimoto, T., Nagata, S., and Tashiro, Y. (1968). Studies on the posterior silk gland of the silkworm, *Bombyx mori*. II. Cytolytic processes in posterior silk gland cells during metamorphosis from larva to pupa. *J. Cell. Biol.* 38:589-603.
- McMahon, T. (1973). Size and shape in biology. Elastic criteria impose limits on biological proportions, and consequently on metabolic rates. *Science.* 179:1201-1204.
- Morimoto, T., Matsuura, S., Nagata, S. and Tashiro, Y. (1968). Studies on the posterior silk gland of the silkworm, *Bombyx mori*. III. Ultrastructural changes of posterior silk gland cells in the fourth larval instar. *J. Cell. Biol.* 38:604-614.
- Nijhout, H. F. and Grunert, L. W. (2002). Bombyxin is a growth factor for wing imaginal disks in Lepidoptera. *PNAS* 99(24):15446-15450.
- Needham, Joseph. (1933). The energy sources in ontogenesis: The respiratory quotient of developing crustacean embryos. *J. Exp. Biol.* 10:79-87.
- Nespolo, R.F., Lardies, M.A., and Bozinovic, F. (2003). Intrapopulational variation in the standard metabolic rate of insects: repeatability, thermal dependence and sensitivity (Q₁₀) of oxygen consumption in a cricket. *J. Exp. Biol.* 206:4309-4315.
- Ohtaki, T., Yamanaka, F., and Sakurai, S. (1986). Differential timing of pupal commitment in various tissues of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 32(7):635-642.
- Pappenheimer, A. M. and Williams, Ca. M. (1953). Cytochrome b₅ and the dihydrocoenzyme I-oxidase system in the cecropia silkworm. *J. Biol. Chem.* 209(2):915-929.

- Parenti, P., Giordana, B., Sacchi, V. F., Hanozet, G. M., Gueritore, A. (1985). Metabolic activity related to the potassium pump in the midgut of *Bombyx mori* larvae. *J. Exp. Biol.* 116:69-78.
- Patel, R.T., Soulages, J. L., Hariharasundaram, B., and Arrese, E. L. (2005). Activation of the lipid droplet controls in the rate of lipolysis of triglycerides in the insect fat body. *J. Biol. Chem.* 280(24):22624-22631.
- Peters, R.H.. (1983). *The ecological implications of body size*. Cambridge. Cambridge University Press.
- Poelman, E.T., Toth, M.J., Ades, P.J., and Calles-Escandon, J. (1997). Gender difference in resting metabolic rate and norepinephrine kinetics in older individuals. *Eur. J. Clin. Invest.* 27(1): 23-28.
- Popjak, G. (1957) Milk fat synthesis from acetate in mammary gland of the cow. *Proc Soc Exp Biol Med.* 21(3):535-53.
- Powers, D.R., and Conley, T.M. (1994). Field metabolic rate and food consumption in two sympatric hummingbird species in southeastern Arizona. *The Condor* 96:141-150.
- Prasad, S. V., Ryan, R. O., Law, J. H. and Wells, M. A. (1986). Changes in lipoprotein composition during larval-pupal metamorphosis of an insect, *Manduca sexta*. *J. Biol. Chem.* 261(2):558-562.
- Ragan, C.I., Wilson, M. T., Darley-USmar, V. M., and Lowe, P. N. (1987). Sub-fractionation of mitochondria and isolation of the proteins of oxidative phosphorylation. In *Mitochondria, a practical approach*. pp.79-113. Oxford-Washington: IRL Press.
- Reynolds, S. E. (1980). Integration of behavior and physiology in ecdysis. *Adv. Insect Physiol.* 15:474-495.
- Rockstein, M. (1973). *The physiology of Insecta*. New York and London. Academic Press.
- Rogowitz, G.L., and Chappell, M.A. (2000). Energy metabolism of eucalyptus-boring beetles at rest and at locomotion: Gender makes a difference. *J. Exp. Biol.* 203: 1131-1139.
- Sakurai, T., Nakagawa, T., Mitsuno, H., Mori, H., Endo, Y., Tanoue, S., Yasukochi, Y., Touhara, K. and Nishioka, T. (2004). Identification and functional characterization of a sex pheromone receptor in the silkworm, *Bombyx mori*. *PNAS* 101(47): 16653-16658.

- Sanborn, R. C. and Williams, C. M. (1950). The cytochrome system in the cecropia silkworm with special reference to the properties of a new component. *J. Gen. Physiol.* 33:579-588.
- Sasaki, S. and Tashiro, Y. (1976). Studies on the posterior silk gland of the silkworm, *Bombyx mori*. V. Electron microscope localization of fibroin in the posterior silk gland at the later stage of the fifth instar. *J. Cell. Biol.* 70(3):648-659.
- Sasaki, S., Nakajima, E., Fujii-Kuriyama, and Y., Tashiro. (1981). Intracellular transport and secretion of fibroin in the posterior silk gland of the silkworm *Bombyx mori*. *J. Cell. Sci.* 50:19-44.
- Sato, Hiroshi. (1976). Changes in respiratory enzyme activities during the early embryonic stages of the silkworm. *Insect Biochemistry.* 6:475-478.
- Sato, H., and Takesue, S. (1975). The cytochrome system of the early embryonic stages of the silkworm. *Insect. Biochem.* 5: 553-562.
- Schmidt-Nielsen, Knut. (1984). *Scaling. Why is animal size so important?* Cambridge. Cambridge University Press.
- Schmidt-Nielsen, K. (2001). *Animal physiology: Adaptation and environment.* Cambridge University Press. USA.
- Schultz, T. W., and Jungreis, A. M. (1977). The goblet cavity matrix in the larval midgut of *Hyalophora cecropia*. *J. Insect Physiol.* 23:29-32.
- Schutz, M.Y. (1997). The effect of obesity, age, puberty, and gender on resting metabolic rate in children and adolescents. *Eur. J. Pedi.* 156(5): 125-129.
- Smith, R. L. (1984). Allometric scaling in comparative biology: problems of concept and method. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 246:152-160.
- Stusek, P., Pohleven, F. and Capl, D. (2000). Detection of wood boring insects by measurement of oxygen consumption. *Int. Biodeter. Biodeg.* 46:293-298.
- Suarez, R.K., Darveau, C.A., Welch, Jr., K.C., O'Brien, D.M., Roubik, D.W., Hochachka, P.W. (2005). Energy metabolism in orchid bee flight muscles: carbohydrate fuels all. *J. Exp. Biol.* 208:3573-3579.
- Suarez, R. K., Lighton, J. R. B., Joos, B., Roberts, S. P., Harrison, J. F. (1996). Energy metabolism, enzymatic flux capacities, and metabolic flux rates in flying honeybees. *PNAS* 93:12616-12620.
- Sutherland, T.D., Campbell, P.M., Weisman, H.E., Trueman, A., and Sriskantha, W. (2006). A highly divergent gene cluster in honey bees. *Gen. Res.* 16:1414-1421.

- Syed, Z., Ishida, Y., Taylor, K., Kimbrell, D.A., and Leal, W.S. (2006). Pheromone reception in fruit flies expressing a moth's odorant receptor. *PNAS* 103(44): 16538-16543.
- Szolajska, E., Poznanski, J., Ferber, M.L., Michalik, J., Gout, E., Fender, P., Bailly, I., Dublet, B., and Chroboczek, J. (2004). Poneratoxin, a neurotoxin from ant venom: Structure and expression in insect cells and construction of a bio-insecticide *Eur. J. Biochem.*, 271:2127 - 2136.
- Tashiro, Y., Morimoto, T., Matsuura, S. and Nagata, S. (1968). Studies on the posterior silk gland of the silkworm, *Bombyx mori*. I. Growth of posterior silk gland cells and biosynthesis of fibroin during the fifth larval instar. *J. Cell. Biol.* 38:574-588.
- Tenney, S.M. (1985). Oxygen supply and limiting oxygen pressures in an insect larva. *Resp. Physiol.* 60:121-134.
- Thomas, M. V. and May, T.E. (1984). Active potassium ion transport across the caterpillar midgut. *J. Exp. Biol.* 108:273-291.
- Tojo, S., Betchaku, T., Ziccardi, V. J. and Wyatt, G. R. (1978). Fat body protein granules and storage proteins in the silkmoth, *Hyalophora cecropia*. *J. Cell. Biol.* 78:823-838.
- Walsberg, G.E., and Hoffman, T.C.M. (2005). Direct calorimetry reveals large errors in respirometric estimates of energy expenditure. *J. Exp. Biol.* 208:1035-1043.
- Wang, Z. Pierson, R.N., and Heymsfield, S.B. (1992). The five level model: a new approach to organizing body-composition research. *Am. J. Clin. Nut.* 56(1):19-28.
- Watanabe, M. I. and Williams, C. M. (1950). Mitochondria in the flight muscles of insects. I. Chemical composition and enzymatic content. *J. Gen. Physiol.* 34: 675-689.
- White, C.R., and Seymour, R.S. (2003). Mammalian based metabolic rate is proportional to body mass $2/3$. *PNAS*. 100: 4046-4049.
- Wieser, W. (1984). A distinction must be made between the ontogeny and the phylogeny of metabolism in order to understand the mass exponent of energy metabolism. *Resp. Physiol.* 55:1-9.
- Weis-Fogh, T. (1964). Diffusion in insect wing muscle, the most active tissue known. *J. Exp. Biol.* 41:229-256.
- West, G.B., Brown, J.H., and Enquist, B.J. (1997). A general model for the origin of allometric scaling laws in biology. *Science*. 276:122-126.

- West, G.B., Brown, J.H. and Enquist, B.J. (2001). A general model for ontogenetic growth. *Nature*. 413:628-631, 2001.
- West, GB, Woodruff, W. H., and Brown, J.H. (2002). Allometric scaling of metabolic rate from molecules and mitochondria to cells and mammals. *PNAS* 1:2473-2478.
- Wood, S. G., Johansen, K., Glass, M. L., and Maloiy, G.M.O. (1978). Aerobic metabolism of the lizard *Varanus exanthematicus*: Effects of activity, temperature, and size. *J. Comp.Physiol.* 127:331-336.
- Zhou, C.Z., Confalonieri, F., Medina, N., Zivanovic, Y., Esnault, C., Yang, T., Jacquet, M., Janin, J., Dugeut, M., Perasso, R. (2000). Fine organization of *Bombyx mori* fibroin heavy chain gene. *Nuc. Acids. Res.* 28:2413-2419.